

DONOR SPECIFIC HYPORESPONSIVENESS

IN

RENAL TRANSPLANT RECIPIENTS

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DECLARATION

I declare that the work contained within this thesis is original and has been composed by me, with any exceptions clearly indicated. This research has been undertaken in Laboratory 301, Department of Immunology, Western Infirmary, Glasgow under the supervision of Professor Allan Mowat and Dr Margaret McMillan. This thesis has not been submitted for any other professional degree.

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ABSTRACT

A phenomenon called donor-specific hyporesponsiveness (DSH) has been described in some renal allograft recipients, in which there is progressive loss of responsiveness to donor tissues and this has been shown to be associated with a favourable long-term outcome. Despite its potential clinical importance, the mechanisms of DSH are unknown and there are no laboratory tests that accurately predict its development in individual patients which might allow the reduction of immunosuppressive therapy. Here I test the hypothesis that renal allograft recipients with DSH can be identified by analysis of the mixed lymphocyte reactivity in-vitro and that this state will be accompanied by the production of inhibitory cytokines such as IL-10 and TGF- β .

The study comprised 78 patients from a single centre, 60 of whom were studied retrospectively and 18 prospectively over a one-year period. DSH was detected by donor specific mixed lymphocyte reactions and cytokine production was analysed by ELISA and PCR. Overall, DSH was found in 61% of cadaveric and 57% of living-related recipients in the retrospective cohort and in 36% of cadaveric and 25% of living-related recipients at one year post-transplant in the prospective cohort. DSH was associated with a lower incidence of late acute rejection in cadaveric and living-related recipients in both arms of the study. Chronic rejection was found in some patients, even in the presence of DSH, indicating that DSH is not exclusive to patients with a good allograft outcome.

DSH correlated with a good graft outcome in long-term cadaveric recipients and was associated with low donor-specific IL-2 and high IL-4 production. Similarly, good graft outcome and DSH was associated with a trend towards low donor-specific IL-2

and high IL-4 production within the first year post-transplant. However, in long-term living-related recipients, DSH did not correlate with graft outcome and was associated only with low IL-2 production. A sub-group of cadaveric recipients with a de-novo solid organ malignancy shared many similar clinical and immunological features with their counterparts who did not have malignancy. This included good graft outcome, low acute rejection rate and a high incidence of DSH. Patients with malignancy produced low levels of IL-2, but also produced high levels of IL-10. However, there was no evidence of immune regulation mediated by IL-10 or TGF- β in any part of the study.

My results suggest that it may be possible to select patients for tailoring of immunosuppression on the basis of detection of DSH, together with the production of a favourable cytokine profile at one year post-transplant. Potential candidates include cadaveric recipients with stable graft function who show DSH and produce four-fold higher levels of donor-specific IL-4 than IL-2, as well as HLA-ID living-related recipients with stable graft function who produce low levels of donor-specific IL-2.

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ABBREVIATIONS

AICD	Activation-induced cell death
APC	Antigen presenting cell
AR	Acute rejection
AZA	Azathioprine
CAD	Cadaveric
CD	Cluster of differentiation
CML	Cell-mediated lympholysis
CR	Chronic rejection
CREAT	Creatinine
CTLA	Cytotoxic T lymphocyte-associated molecule-4
CYC	Cyclosporin
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide triphosphates
DSH	Donor-specific hyporesponsiveness
DSR	Donor-specific reactivity
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
EBV	Ebstein Barr virus
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay

FcR	Fragment, crystalline receptor
FCS	Foetal calf serum
F'N	Function
HCl	Hydrochloric acid
HI	Haplo-identical
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule 1
ID	Identical
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LDA	Limited dilution assay
LFA-1	leukocyte function antigen-1
LRD	Living-related donor
M/M	Mismatch
MgCl ₂	Magnesium chloride
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
MMF	Mycophenolate mofetil
NI	Non-identical
NK	Natural killer
OKT3	Muromonab-CD3
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffed saline
PCD	Passive cell death
PCR	Polymerase chain reaction
pCTL	Precursors for cytotoxic T cells
PD	Programmed death
PHA	Phytohaemmagglutinin
pHTL	Precursors for helper T cells
PRA	Panel reactive antibody
PRED	Prednisolone
PTLD	Post-transplant lymphoproliferative disorder
R	Receptor
ROC	Receiver operating characteristic
rpm	revolutions per minute
RPMI	Roswell park memorial institute
RRI	Relative response index
RT	Reverse transcription
SI	Stimulation index
S-R	Steroid resistant
S-S	Steroid sensitive
TAC	Tacrolimus
TcR	T cell receptor
TGF- β	Transforming growth factor - beta
Th	T helper
TMB	Tetra-methyl-benzidine

T-reg	Regulatory T cell
T _R 1	Regulatory T cell-1
TX	Transplant
DX	Diagnosis

ABSTRACTS

1. Alfonzo, A.V.M. McMillan, M.A. Briggs, J.D. Mowat, A.McI. De-novo solid organ malignancy following renal transplantation is associated with donor-specific hyporesponsiveness and IL-10. **British Transplantation Society – Oxford, March 2001.**
2. Alfonzo, A.V.M. Mowat, A.McI. McMillan, M.A. Briggs, J.D. Rodger, R.S.C. and Junor, B.J.R. The role of cytokines in the development of donor-specific hyporesponsiveness in renal transplant recipients. **British Society of Immunology - Harrogate, December 2000.**
3. Annette Alfonzo on behalf of the Renal Unit, Western Infirmary, Glasgow. Immunological outcome of patients with de-novo solid organ malignancy following renal transplantation. **Scottish Renal Association – Inverness, November 2000.**
4. Alfonzo, A.V.M. Mowat, A.McI. McMillan, M.A. Briggs, J.D. Rodger, R.S.C. and Junor, B.J.R. The role of cytokines in the development of donor-specific hyporesponsiveness in renal transplant recipients. **American Society of Nephrology - Toronto, October 2000.**
5. Alfonzo, A.V.M. Mowat, A.McI. McMillan, M.A. Briggs, J.D. Rodger, R.S.C. and Junor, B.J.R. The role of cytokines in the in-vitro development of donor-specific hyporesponsiveness in renal transplant recipients. **Scottish Renal Association – Dundee, May 2000.**

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Immunological outcome of patients with de-novo solid organ malignancy following renal transplantation.

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De-novo solid organ malignancy following renal transplantation is associated with donor-specific hyporesponsiveness And IL-10. Alfonzo A V M, McMillan M A, Briggs J D and Mowat A McI.

British Society of Immunology – December 2000

The role of cytokines in donor specific hyporesponsiveness after renal transplantation. Alfonzo A V M, McMillan M A, Briggs J D and Mowat A McI.

American Society of Nephrology – October 2000

Patients with de-novo solid organ malignancy following renal transplantation show donor-specific hyporesponsiveness and a Th2 Bias. Alfonzo A V M, Briggs J D, McMillan M A and Mowat A McI.

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DEDICATION

I dedicate this thesis to my husband, Bryne, who has provided endless support and encouragement over the years and to my son, Kieran, who has put up with my absence while writing.

CHAPTER 1: GENERAL INTRODUCTION

OBJECTIVES

Renal transplantation has significantly improved the long-term survival and the quality of life for many patients with end-stage renal failure and has become the preferred mode of renal replacement therapy. However, the morbidity associated with long-term immunosuppression remains an important problem. Although specific immune tolerance to donor alloantigens can readily be induced and maintained in animal models (Larsen et al, 1996), this has been difficult to achieve in humans. Nevertheless, in some non-compliant patients and in some of those with malignancy, excellent graft function can be maintained despite little or no immunosuppression (Reyes et al, 1993; Ishido et al, 1999; Birkeland et al, 2000). This suggests that it may be possible to reduce the amount of immunosuppression in some recipients without inducing rejection episodes and it would be useful to be able to identify such patients. To achieve this, a reliable method is needed to monitor immune responses in vitro. Although many strategies have been proposed for this purpose, few have been used successfully to guide clinical decisions and there remains no reliable immunological assay that can be used safely to tailor immunosuppression.

A phenomenon known as donor-specific hyporesponsiveness (DSH) has been described in which the allograft recipient shows reduced reactivity or non-reactivity towards donor cells, but retains normal reactivity towards other antigens (Bach et al, 1968; Fletchner et al, 1984; Reinsmoen et al, 1990). Despite its potential clinical importance, the mechanism by which DSH occurs remains unknown. There is

increasing evidence that differential production of cytokines by T cells may be important in a variety of models of immunological tolerance, but the possibility that this is associated with DSH has not been widely studied. The purpose of my work was to develop an immunological method to guide reduction of immunosuppression by assessing donor-specific proliferative responses and cytokine production in recipients of renal allografts. I tested the hypothesis that renal allograft recipients with good graft outcome would show DSH and that this state would be accompanied by the production of inhibitory cytokines such as IL-10 and TGF- β .

COMPLICATIONS OF RENAL TRANSPLANTATION

Transplantation is not without risk. Early post-operative complications following renal transplantation may be surgical, medical or immunological. These include haemorrhage, urinary leaks, delayed graft function, sepsis and acute rejection. As graft survival has improved in recent years, the long-term medical complications of transplantation have become increasingly important. The most important of these include cardiovascular disease, infection, bone disease, and drug-induced diabetes (Kasiske et al, 2000; Wheeler et al, 2000; Ojo et al, 2000). Malignancy is a further, highly feared complication, as it often necessitates a reduction of immunosuppression, sometimes at the expense of graft loss. Death of patients with functioning grafts accounts for more than 50% of late graft loss and in the surviving patients, chronic rejection is the commonest cause of graft failure (Joseph et al, 2001).

Current strategies to prevent late graft loss include improved peri-operative management, prevention and treatment of acute rejection, discontinuation of steroids in stable patients and the assessment and treatment of cardiovascular risk factors. The induction and identification of donor-specific hyporesponsiveness to allow minimal immunosuppression or the induction and maintenance of tolerance requiring no maintenance immunosuppression is one strategy of possible future importance (Pascual et al, 2002). It is this aspect which my study focussed on.

ACUTE REJECTION

Acute rejection may be defined as an unexplained rise in serum creatinine, occurring most frequently within the first three months post-transplant, that is confirmed on biopsy or that responds to treatment with corticosteroids (Massy et al, 1995). It is a T lymphocyte mediated immune response characterised by a mononuclear interstitial infiltrate with tubulitis and varying degrees of vascular inflammation (Almond et al, 1993). In the acute rejection process, T cell activation occurs predominantly via the direct pathway of allorecognition, whereby the recipient's T cell receptors (TcR) recognise intact MHC molecules on the surface of donor cells. This is induced predominantly by professional antigen presenting cells (APC) such as dendritic cells (DC) which are abundant in the graft during the early post-transplant period, and play a key role in antigen-processing and presentation to T cells. Over time, the number of donor-derived APC in the graft decrease leading to a diminution of the incidence and severity of acute rejection (Allan et al, 2002). This is reflected in clinical practice by the observation that the course of a renal transplant is characterised by an early high probability of acute rejection, followed by a period

when acute rejection is less common despite lower immunosuppression (Gourishankar et al, 2001).

Most immunosuppression regimens aim to reduce the incidence of acute rejection with the anticipation that this would improve long-term graft survival. Before the introduction of azathioprine in the 1960's, most grafts were lost in the first year post-transplant as a result of acute rejection. The introduction of cyclosporin in the mid-1980's led to a further improvement in short-term graft survival, but it did not solve the problem of late graft loss and it is too early to determine whether newer immunosuppressive agents will address this issue. A recent multivariate analysis has shown a 21% difference in 5-year graft survival between patients with and without a history of acute rejection, suggesting that it is still important to reduce the incidence of acute rejection (Allan et al, 2002).

Acute rejection increases the risk of developing chronic rejection and subsequent graft loss. Many factors influence this effect of acute rejection on graft outcome, in particular the timing, number and severity of acute rejection episodes. Acute rejection within the first three months post-transplant may have no effect on chronic rejection (Massy et al, 1996), but after three months confers the greatest risk (Humar et al, 1999; Joseph et al, 2001). Indeed, patients with no or only early acute rejection have been shown to have significantly better long-term graft survival than patients who had acute rejection after the first three months post-transplant (Joseph et al, 2001; Sijpkens et al, 2003). Patients who experience multiple acute rejection episodes also have a significantly increased risk of subsequent graft loss (Basadonna et al, 1993; Monaco et al, 1999; Humar et al, 2000). Severe acute rejection with vascular injury exerts a more detrimental effect on graft survival than those that

result in complete functional recovery (Massy et al, 1996; Mueller et al, 2000). Therefore, long-term graft outcome is dependent both on preventing acute rejection and on reducing the likelihood of subsequent episodes, both of which are largely influenced by HLA-matching and optimising immunosuppression. Hence in my study, good graft outcome was defined on the basis of no or only early steroid responsive acute rejection occurring within the first three months post-transplant and the maintenance of good graft function.

CHRONIC REJECTION

Chronic rejection is the slow, insidious and progressive deterioration of renal function, as evidenced by increasing serum creatinine levels, and generally occurs more than 90 days post-transplantation (Monaco et al, 1999). Despite modern immunosuppression and surgical techniques, the incidence of graft loss beyond the first year remains unchanged at a rate of about 5% each year post-transplantation (Cecka, 1999; Keck et al, 1999). The immune mechanism of the chronic rejection process is not fully understood, but allorecognition appears to occur predominantly via the indirect pathway, whereby alloantigens are processed by recipient APC and the resulting peptide antigens are presented to host T cells in the context of self MHC molecules. As virtually all the parenchymal cells of an allograft can provide a source of antigens for processing by host APC, indirect allorecognition is a potentially ever-present mechanism that may sustain long-term alloresponsiveness (Allan et al, 2002). Many non-immunological factors have also been implicated in the aetiology of chronic rejection, including hypertension, cyclosporin nephrotoxicity and

cytomegalovirus infection, all of which may promote vascular injury (Almond et al, 1993; Monaco et al, 1999; Allan et al, 2002).

The criteria for the diagnosis of chronic rejection are not standardised, but the gradual deterioration in graft function is often associated with proteinuria and hypertension, together with typical biopsy findings of interstitial fibrosis, glomerular sclerosis and fibrointimal proliferation in intra-renal arteries. Although there is some evidence that mycophenolate mofetil can stabilise graft function in patients with chronic rejection (Weir et al, 1997), most patients invariably progress to end-stage renal failure. As little can be done to modify the immune process at present, much attention is paid to addressing the non-immunological risk factors for chronic rejection in clinical practice. I have studied a group of patients with poor graft outcome, defined on the basis of biopsy-proven chronic rejection or risk factors for chronic rejection such as late, multiple or severe acute rejection, to compare the incidence of DSH and donor-specific cytokine production in patients without chronic rejection.

MALIGNANCY

There is an increased risk of malignancy post-transplantation in patients with end-stage renal failure, with an incidence estimated to be 3- to 4-fold greater than that of age-matched controls in the general population (Penn, 1998). This is not seen to the same extent in patients receiving long-term haemodialysis (Birkeland, 2000). Several mechanisms have been proposed for the high incidence of malignancy associated with renal transplantation, including defective immune surveillance as a result of immunosuppression leading to increased susceptibility to virally induced

malignancies such as Kaposi's sarcoma and cervical carcinoma (Sheil et al, 1991). Alternatively, some immunosuppressive drugs themselves may be oncogenic, while agents such as anti-thymocyte globulin and OKT3 which deplete T lymphocytes may lead to secondary lymphoid hyperplasia and potential dysplasia, thereby increasing the risk of development of post-transplant lymphoproliferative disorders (PTLD). The total cumulative immunosuppressive dose is well correlated with the development of malignancy, and patients treated with cytolytic therapy for treatment of acute rejection are particularly at risk (Silkensen, 2000).

PTLD is a life-threatening complication of transplantation which is dependent on exposure to the Epstein-Barr virus (EBV), and on the type and duration of immunosuppression. To my knowledge, this is the only post-transplant malignancy in which donor reactivity in MLR and donor-specific cytokine production has been assessed. Birkeland et al (2000) studied the role of IL-10 in the development of EBV-associated PTLD and proposed that IL-10 may act in an inhibitory capacity to promote viral infection and cell transformation by reducing NK cell control over B-cell proliferation, and suggested that IL-10 may also account for the operational tolerance observed in some patients.

All patients undergoing renal transplantation are made aware of the potential risk of malignancy and efforts are routinely made to reduce this risk by careful screening of recipients and close monitoring post-transplantation. Nevertheless, some patients develop tumours and in the immunosuppressed state, this is frequently associated with reduced survival. I studied patients who developed a malignancy post-transplant, as it has been observed that the reduction of immunosuppression in some patients with malignancy did not lead to acute rejection or graft loss. Hence, I

wished to test the hypothesis that these patients would be one group that would show donor-specific hyporesponsiveness and produce inhibitory cytokines in response to donor stimulation.

IMMUNOSUPPRESSION AND ASSOCIATED PROBLEMS

Much of the morbidity following renal transplantation has been attributed to long-term immunosuppression. Furthermore, immunosuppressive therapy has its own limitations, such as drug toxicity and inter-individual variations in sensitivity to the drugs. The most commonly used immunosuppressants are corticosteroids, azathioprine and cyclosporin. In clinical practice, these drugs are used as a triple therapy and they work synergistically, as they interfere with different stages of the rejection process. Corticosteroids play an important role in the prevention and treatment of allograft rejection, but they are also responsible for a significant amount of post-transplant morbidity. Long-term steroid therapy may cause a Cushingoid appearance, bone disease, diabetes, hyperlipidaemia and adrenal suppression. Azathioprine is a purine analogue that is incorporated into cellular DNA where it inhibits purine nucleotide synthesis and consequently T-cell activation. Its most important side effect is bone marrow suppression. Cyclosporin, a calcineurin inhibitor which interferes with IL-2 gene transcription, thereby inhibiting T-cell activation, has improved 1-year allograft survival from 50% to greater than 80%, but can cause nephrotoxicity and ultimately allograft dysfunction. It may also cause hypertension, diabetes, gum hypertrophy and hyperkalemia. Therefore, it is not

surprising that non-compliance with complex and prolonged therapy is a problem and may adversely affect graft outcome (Gaston et al, 2001).

Combination therapy is used to allow lower doses of individual drugs to be used, thereby reducing side-effects. Tailoring of immunosuppression to reduce these adverse outcomes without the guidance of immunological assays has proven to be hazardous and highlights the need for on-going research. Although cyclosporin has allowed lower maintenance doses of steroids to be used, discontinuation of steroids is successful in only two-thirds of patients and the increased risk of rejection is generally thought to be unacceptable (Hricik et al, 1994, 2002; Ratcliffe et al, 1996; Dunn et al, 1999). These agents are being increasingly replaced by newer drugs that are more selective and may have fewer side effects. Tacrolimus, mycophenolate mofetil, sirolimus, and humanised anti-IL-2 receptor monoclonal antibody therapy undoubtedly improve short-term graft survival, but it is still too early to determine their impact on long-term outcome.

TOLERANCE INDUCTION STRATEGIES

Transplant tolerance may be defined as the stable long-term engraftment of an organ in the absence of immunosuppression in a recipient who remains immunocompetent (Allan et al, 2002). The reduced immune response is specific to the donor antigens and it appears to be an active process (Kirk et al, 2000). The pioneering work of Medawar and colleagues (1953), provoked intense study of transplantation tolerance, as it suggested that long-term graft survival could be attained by inducing specific

tolerance to donor allo-antigens. This would theoretically reduce the need for high dose immunosuppression and hence alleviate the problems of drug toxicity.

Many different approaches have been tried to induce tolerance in experimental models. Historically, recipients of pre-transplant blood transfusion have shown long-term graft survival in both experimental (Fabre et al, 1972; Wood et al, 1985) and clinical transplantation (Morris et al, 1968; Opelz et al, 1973). The timing, HLA-match and the systemic administration of antigen all appear to contribute to donor-specific unresponsiveness after transfusion (Starzl et al, 1996; Lapchak et al, 2002). However, the introduction of cyclosporin led to the decline in the use of donor-specific blood transfusion as the benefit to graft survival was less notable and possible sensitisation of recipients pre-transplant was a concern (Potter et al, 1991). Successful engraftment of a solid-organ transplant has been achieved without immunosuppression when accompanied by a bone marrow transplant from the same donor and this appears to be facilitated by chimerism of donor haematopoietic cells in the recipient (Sayegh et al, 1991; Helg et al, 1994; Hamawi et al, 2003). However, the myeloablative treatment needed for this procedure can only be justified in patients who require bone marrow transplantation for a primary haematological disease.

Other methods of inducing unresponsiveness to MHC-mismatched allografts at the time of transplantation involve lymphocyte depletion which impairs the primary immune response but allows a gradual repopulation of the lymphoid compartment. This seems to be associated with the development of immunoregulatory mechanisms that maintain tolerance to antigens present at the time (Kanmaz et al, 2003). In this way, immunological unresponsiveness becomes

allo-specific, despite the non-specific nature of the initial lymphocyte depletion. Depletion strategies include total lymphoid irradiation (Strober et al, 1989, 2000; Reinsmoen et al, 1991) and polyclonal anti-lymphocyte globulin (Burk et al, 1997). Other more specific strategies include the use of monoclonal antibodies that block critical ligands in the T cell-APC interaction, such as anti-CD2 (Kaplon et al, 1996; Bai et al, 2002), anti-CD3 (Nicolls et al, 1993; Burk et al, 1997), and anti-CD4 (Cobbold et al, 1992, 1996; Bushell, 1994; Saitovitch et al, 1995; Hall et al, 2000). Analogous approaches include the blockade of the costimulatory molecules B7 and CD28 by the use of CTLA-4 Ig (Pearson et al, 1994), blockade of CD40 ligand by anti-CD154 (Parker et al, 1995; Xu et al, 2002), or a combination of CD28 and CD40 blockade (Pearson et al, 1996). Alternatively, antibodies directed against leukocyte function antigen-1 (LFA-1) (Houmant et al, 1996), intercellular adhesion molecule (ICAM) 1 (Haug et al, 1993), or the IL-2R (Nashan et al, 1997; Vincenti et al, 1998) also appear to protect against acute rejection.

Although the initial depletion of allo-reactive cells at the time of transplantation can favour long-term unresponsiveness, inhibition of newly produced allo-reactive T cells is required to maintain unresponsiveness (Kanmaz et al, 2003). Other problems associated with tolerance induction protocols include toxicity, infectious complications and the risk of malignancy. Additionally, many of these strategies have been successful in animal models, but have been disappointing in clinical trials. Therefore, the safety and efficacy of these strategies limit their use in clinical practice and require further study with longer patient follow-up.

Tolerance induction strategies will only be clinically useful if there are means of monitoring allo-responsiveness. A variety of ways of doing this have been

described, including assessing direct and indirect T cell alloreactivity by proliferation, cytokine production, in vivo delayed-type hypersensitivity (DTH), graft morphology and immunohistochemistry of graft infiltrating cells (Salama et al, 2001). Although some of these assays allow monitoring of the graft-specific immune response, accurate methods to measure tolerance and their clinical validation are still lacking. In particular, proliferation assays have been criticised for their lack of reproducibility and some have shown no correlation with graft outcome. The value of in vivo DTH assays is uncertain, as only few studies have been performed using this method. The assessment of graft morphology and detection of staining for cytokine protein in graft infiltrating cells by immunohistochemistry require invasive procedures which are not without risk. Cytokine analysis using techniques such as ELISA, ELISPOT and flow cytometry have all been tried in small studies, but have not shown a correlation with graft outcome (Reinsmoen et al, 2002).

As immune responsiveness and immune regulation are dynamic processes, it is likely that more than one method will be required to assess the recipient's immune status and serial analyses will be required (Salama et al, 2001; Reinsmoen et al, 2002). An approach involving more than one assay will also provide reassurance to the clinician who is ultimately responsible for the consequences of adjusting treatment, at least until a single test proves to be reliable and safe. Although tolerance induction and tailoring of immunosuppression strategies both require the guidance by immunological parameters, a reliable functional in vitro assay that gives quantitative and qualitative assessment of the recipient-donor reactivity is not yet available.

MECHANISMS OF ANTIGEN-SPECIFIC TOLERANCE

The mechanisms responsible for transplant tolerance are contentious, but are likely to be similar to those that have been implicated in other models of peripheral T cell tolerance. These include clonal anergy or deletion of donor-specific T cells, and the effects of regulatory T cells that can suppress the function of effector T cells via cell-cell contact or the production of cytokines such as IL-4, IL-10 or TGF- β .

CLONAL ANERGY

Clonal anergy is the functional inactivation of antigen-specific T cells. It was first demonstrated by Lamb et al (1983), who showed that T cell clones that were first exposed to antigen on other T cells in vitro were unable to proliferate normally when subsequently exposed to antigen on normal APC. The critical effect of anergic T cells is a failure to proliferate, to act as effector cells or to produce IL-2. In addition, anergic T cells are not merely passive, but may play an important role as regulatory cells (Lechler et al, 2001).

Many mechanisms have been proposed to explain the induction of anergy. Early investigators showed that it may arise following primary antigenic stimulation in the presence of APC that lack appropriate costimulatory signals such as CD28-B7. This leads to a partial primary response by T cells, followed by a long-lasting state of unresponsiveness to challenge via the TcR (Jenkins et al, 1987; Quill et al, 1987). Cell division during the primary response also appears to be important in maintaining subsequent T cell reactivity, as inhibition of cell cycle progression by anti-IL-2 or anti-IL-2R (DeSilva et al, 1994), or with the immunosuppressive agent rapamycin which blocks IL-2R signalling (Powell et al, 1999) can induce T cell anergy, even in

the presence of costimulation. Although anergy in CD4⁺ Th1 cells can be reversed by the addition of exogenous IL-2, anergic suppressive CD4⁺CD25⁺ cells can be expanded by IL-2 (Frasca et al, 1997; Witzke et al, 2001).

Anergy may also be induced by peptide antigens that are partial agonists for the TcR (Lechler et al, 2001). These fail to stimulate primary T cell proliferation, but induce T cell anergy, even when the APC provides full co-stimulation. Thus, it appears therefore that any factor which interferes with the overall affinity of the T cell for its APC during the primary response to antigen may result in anergy. Humanised non-FcR-binding antibodies against CD3 are promising immunosuppressive agents that induce anergy by acting as partial agonist ligands of the TcR and may overcome the adverse effects of conventional monoclonal antibodies against CD3 used in the treatment of acute rejection (Chau et al, 2001; Meijer et al, 2001). Non-FcR-binding antibodies may also prime T cells for AICD upon antigen re-challenge (Yu et al, 2000) and may down-regulate Th1 cytokines, while upregulating Th2 cytokines (Smith et al, 1998).

Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) is a cell surface molecule that is expressed on CD4⁺ and CD8⁺ T cells after T cell activation (McCoy et al, 1999; Sansom et al, 2000) and may play a preferential role in the induction of anergy. CD28 is a T cell surface molecule which delivers a co-stimulatory signal upon interaction with B7 molecules present on APC (McCoy et al, 1999). Inhibitory signals delivered by CTLA-4, which has a higher affinity for B7 than CD28, oppose TcR signalling (Sansom et al, 2000; Kirken et al, 2002) and several mechanisms have been proposed for this inhibitory effect. CTLA-4 signalling blocks IL-2 production, IL-2R expression and cell cycle progression of activated T cells at a very

early stage in T cell activation (Krummel et al, 1996; McCoy et al, 1999). In addition to favouring the development of T cell anergy (Greenwald et al, 2001) CTLA-4 may also be involved in the function of CD4⁺CD25⁺ regulatory T cells (Hara et al, 2001). In contrast, blockade of CTLA-4 leads to enhanced T cell proliferation. This is most clearly demonstrated in CTLA-4 knockout mice that develop fatal lymphoproliferative disease (Waterhouse et al, 1995).

Other newly identified costimulation molecules belonging to the CD28-B7 family may also be involved in regulating T cell responses. PD1, which resembles CD28 and CTLA-4, is expressed on activated T, B and myeloid cells and has two recognised ligands called PDL-1 and PDL-2 (Bernard et al, 2002). The PD-1 pathway is also an inhibitory pathway that acts synergistically with CTLA-4 to help switch off T cell responses against non-self antigens by inducing expression in DC upon their activation (Bernard et al, 2002). Therefore, it appears that strategies which promote CTLA-4 expression while down-regulating CD28 costimulation may be clinically desirable.

CLONAL DELETION

Deletion of antigen-reactive T cells in the thymus has been shown to be the most important mechanism for the induction of tolerance to self-antigens during the development of the immune system (Kappler et al, 1987). Clonal deletion may also play a role in peripheral tolerance, which occurs after mature T lymphocytes have emigrated from the thymus. T cell death via apoptosis is generally considered to be the mechanism of clonal deletion and two mechanisms have been described - activation-induced cell death (AICD) and passive cell death (PCD) (Li et al, 2001).

AICD refers to the susceptibility of activated T cells to undergo apoptosis when high levels of IL-2 production induce expression of the death receptors, Fas and FasL, on activated T cells. PCD occurs when activated effector cells are deprived of growth factors such as IL-2. Li et al (2001) have shown that IL-2 dependent AICD plays a critical role in the development of allograft tolerance following islet cell transplantation and similar findings were reported by another group following murine cardiac allograft transplantation (Dai et al, 1998).

ACTIVE REGULATION

CD4⁺ T cells are central to the development of both allograft rejection and the induction of tolerance (Nickerson et al, 1994, 1997; Field et al, 1997; Zelenika et al, 2001; Waldmann et al, 2001). Infectious tolerance was one of the first indications that CD4⁺ T cells can be induced to have a regulatory function. Waldmann et al demonstrated this phenomenon, whereby regulatory T cells that developed after anti-CD4 antibody therapy can enable other T cells to become tolerant and to have regulatory function (Qin et al, 1993; Waldmann et al, 2001). Anti-CD4 is not the only target immunosuppressive therapy that may result in infectious tolerance. Blockade of CD28-B7 by CTLA-4 (Onodera et al, 1997) and anti-CD40L combined with anti-CD8 (Honey et al, 1999) can also induce this state.

Mosmann et al (1986) first demonstrated that CD4⁺ T cells may produce distinct profiles of cytokines and suggested that there may be different functional subsets. The two T helper cell subsets, Th1 and Th2, are derived from a common Th0 precursor and the microenvironment influences whether Th0 cells will polarise into a Th1 or Th2 cell. The principal early event in Th1 differentiation is the

production of IL-12 by macrophages and dendritic cells in response to inflammation or pathogen products (Trinchieri et al, 1995). Th1 cells produce IL-2 and IFN- γ which promote cellular cytotoxicity and delayed-type hypersensitivity reactions. Th0 cells differentiate into Th2 cells in the presence of IL-4 and produce IL-4, IL-6, IL-9, IL-10 and IL-13 and support humoral immune responses. Th1 and Th2 cytokines are reciprocally antagonistic since IFN- γ inhibits the proliferation of Th2 cells, while IL-10 inhibits IL-12 production and the differentiation of Th1 cells (Coffman et al, 1997).

It has become apparent that other CD4⁺ T-cell subsets which do not conform to a classical type 1 and 2 cytokine profile exist and may play an integral role in the regulation of many aspects of T cell function. In particular, a number of different regulatory T cells (T-reg) have been described in different forms of tolerance, including oral tolerance (Weiner et al, 1994), autoimmunity (Sakaguchi et al, 2000), inflammatory bowel disease (Groux et al, 1997, 1999), and transplantation (Hall et al, 1985; Qin et al, 1993; Zhai et al, 1999; Waldmann et al, 1998, 2000). Th3 cells were first identified by Weiner et al (1994) in studies using oral administration of protein antigens to suppress autoimmune disease. These inhibit other T cells by secreting TGF- β . A further subset of T_R1 cells has been described which is generated in vitro in the presence of IL-10 and produces IL-10 preferentially (Groux et al, 1997). These inhibit antigen-specific T cell responses via inhibition of IL-2 and IL-2R expression (Groux et al, 1996) and by the suppression of APC function (Groux et al, 1998). Much attention has focused recently on regulatory CD4⁺ T cells which express high levels of CD25. These appear to arise spontaneously in the thymus and are exported to the periphery where they function as potent suppressor

cells in preventing T cell responses to self antigens (Shevach et al, 2000). The precise mechanisms underlying their suppressor activity is controversial, but it requires stimulation via the TcR and a variety of factors have been suggested, including IL-10, TGF- β (Dieckmann et al, 2002; Jonuleit et al, 2002) and CTLA-4 (Kingsley et al, 2002). Cell-cell contact seems to be essential and the suppressed cell shows inhibition of IL-2 and IFN- γ gene transcription. The suppressive effect can be overcome by the addition of exogenous IL-2 (Thornton et al, 2000). In addition to spontaneously arising regulatory CD4⁺CD25⁺ T cells, it may also be possible to steer an uncommitted T cell toward regulatory function by repetitive stimulation with immature dendritic cells, or by the presence of regulatory cytokines such as IL-10 and TGF- β (Garin et al, 2003).

T-reg cells may affect the functional activity of other T cells directly, or indirectly by modifying the function of APC. T-reg cells can inhibit proliferation and cytokine production, eliminate effector cells by promoting apoptosis, facilitate the induction of anergy, or convert effector T cells to a regulatory phenotype (Wood et al, 2003). There is also evidence from in vitro studies that T-reg cells can down-regulate the expression of co-stimulatory molecules, such as CD80 and CD86, thereby rendering APC less able to trigger T cell activation (Cederbom et al, 2000; Taams et al, 2000). T-reg cells have also been shown to prevent graft rejection by impairing the function of CD8⁺ T cells (van Maurik et al, 2002; Lin et al, 2002). Active regulation is now accepted to be important in the induction and maintenance of specific immune unresponsiveness to alloantigens following both organ (Hara et al, 2001; Kingsley et al, 2001) and bone marrow transplant (Hoffman et al, 2002) in vivo. Furthermore, a balance between regulation and deletion of responder T cells

has been shown to be an effective strategy in controlling responsiveness after transplantation (Wood et al, 2001; Wells et al, 2001).

The mechanisms of action of Treg remain to be established. It has been widely proposed that a shift towards a Th2 cytokine profile might support allograft tolerance (Sayegh et al, 1995; Chen et al, 1996a; Onodera et al, 1997), while a predominance of a Th1 profile may lead to allograft rejection (Mottram et al, 1995; McLean et al, 1997; D'Elios et al, 1997). Infectious tolerance after blockade of CD4 may be dependent on Th2 cytokines, particularly IL-4, *in vivo* (Davies et al, 1996; Onodera et al, 1997; Bushell et al, 1999), and on IL-10 and TGF- β *in vitro* (Dieckmann et al, 2002). These regulatory CD4⁺ T cells can also mediate linked suppression, whereby T cells specific for an unrelated third party antigen can be tolerised if both tolerising and third party antigens are presented together on the same graft (Davies et al, 1996; Chen et al, 1996b). Early studies showed that high IL-10 production was associated with skin, cardiac and islet graft acceptance (Hancock et al, 1993) and with the induction of neonatal and adult tolerance to foreign antigens (Bacchetta et al, 1994). Many tolerance induction regimens have also shown a predominance of Th2 cytokines (Sayegh et al, 1995; Zeng et al, 1996; Field et al, 1997). Further evidence for a role of Th2 cytokines in graft acceptance has come from studies in liver transplantation, where high levels of donor-specific IL-4 and IL-10 have been found to be strongly associated with stable liver allograft function independent of the level of immunosuppression, while patients who develop acute rejection produce high levels of IFN- γ (Chung et al, 1998; Minguela et al, 1999). These findings may help explain why the liver appears to be less immunogenic than the kidney following allo-transplantation. However, other investigators have not

supported the hypothesis of a Th2 bias in allograft tolerance, as both Th1 and Th2 cytokines may be expressed within the graft during acute rejection and tolerance can be observed in the absence of Th2 cytokines (Dallman et al, 1995; Picotti et al, 1997; Kuzendorf et al, 1998).

Therefore, it is unclear how Th2 cells contribute to tolerance, or if they simply arise as a result of the tolerant state. Disappointingly, attempts to induce tolerance either by enhancing the development of Th2 cells in vivo (Chan et al, 1995; VanBuskirk et al, 1996; Picotti et al, 1996), or by the systemic administration of IL-4 and/or IL-10 (Vossen et al, 1995; Qian et al, 1996) have failed to prolong graft survival. The conflicting evidence for the relative contributions of Th1 and Th2 cytokines in graft acceptance and rejection may reflect variations in inductive strategies, the organ involved, and whether the graft was immediately vascularised or not. Furthermore, many of the early studies relating cytokine production following transplantation have been based on the expression of cytokine genes in the grafts, or by identifying graft infiltrating cells that stain for cytokine protein by immunohistochemistry which do not always reflect antigen-specificity (Picotti et al, 1997).

Graft acceptance has also been found in association with donor-specific IL-10 and TGF- β production. TGF- β is a cytokine with immunoregulatory properties and can control the differentiation, proliferation and state of activation of several immune cells (Letterio et al, 1998). Induction of allo-specific tolerance by transfusion of donor cells has been shown to be associated with high levels of TGF- β mRNA and protein, and it can be reversed by the administration of anti-TGF- β monoclonal antibody (Josien et al, 1998). Furthermore, apoptosis of allo-reactive cells can induce

production of IL-10 and TGF- β (Gao et al, 1998). Although cytokines are important in immunoregulation, it is likely that the pathway to allograft tolerance involves more than one of the mechanisms described above. This is evidenced by the findings that clonal deletion and immune deviation may occur simultaneously in neonatal tolerance (Matriano et al, 1994; Donckier et al, 1995), as well as in other models of tolerance (Gao et al, 1998), and that otherwise anergic T cells may act as regulatory cells (Lechler et al, 2001).

MICROCHIMERISM

Microchimerism is the persistence of donor-derived haematopoietic cells, particularly dendritic cells, in recipient tissues many years after transplantation (Starzl et al, 1992, 1993; Larsen et al, 1990; Burlingham et al, 1996). Numerous reports have supported the idea that microchimerism is the mechanism of tolerance induced by donor-specific blood transfusion (Flye et al, 1995) or allogeneic bone marrow transplantation (Dey et al, 1998; Butcher et al, 1999; Hamawi et al, 2003). Early development and long persistence of microchimerism has also been suggested as one reason why liver allografts are relatively resistant to rejection compared with other organs (Starzl et al, 1992; Lu, 1995). However, the exact role of microchimerism remains controversial and some workers have found no evidence of microchimerism in patients who show operational tolerance (Bushell et al, 1995; Fuchinoue et al, 2002). In addition, there is debate over the most appropriate methods of detection, the anatomical sites in which it is found (Noris et al, 2001), as well as its clinical relevance (Strober et al, 2000). Although the persistence of allo-antigens appears to be essential for most of the other mechanisms implicated in the

development of tolerance (Khan et al, 1996), it may be the graft itself, and not microchimerism, that is crucial for the maintenance of tolerance in vivo (Hamano et al, 1996; Karim et al, 2002; Wood et al, 2003).

DSH

Donor-specific hyporesponsiveness was first described by Amos and Bach, when they demonstrated that lymphocytes from certain recipients of allogeneic kidneys showed uni-directional unresponsiveness to donor cells in mixed lymphocyte culture (Amos et al, 1968). Later, Garovoy et al (1973) showed that all recipients who were 8 to 12 years post-transplant were non-responsive to donor stimulation and Thomas et al (1977) demonstrated a trend toward lower donor-specific responses in patients 8 to 12 years after transplant compared with those 2 to 5 years post-transplant. However, these early studies were hampered by the fact that HLA antigens, particularly Class II HLA antigens, were poorly defined at that time, and by the use of patients' sera in the culture medium. Although DSH may not necessarily signify true antigen-specific tolerance, as most patients are immunosuppressed and some patients who show DSH develop acute rejection following withdrawal of immunosuppression, it does represent a reduced reactivity to donor cells. As it is unethical to discontinue immunosuppression for the purpose of immunological study, DSH appears to be the closest marker of tolerance in clinical transplantation. As DSH has also been described in patients who maintain their grafts without immunosuppressive therapy, it seems likely that DSH reflects antigen-specific tolerance and not a complication of immunosuppression.

More recently, prospective studies have been performed to assess how DSH develops after transplantation. In cadaveric recipients followed for up to 4 years, the reported incidence of DSH ranged widely from 25-74% (Bas et al, 1992; Reinsmoen et al, 1993; Ghobrial et al, 1994; Creemers et al, 1997; Kerman et al, 1997). There are many factors which may explain this large variation in incidence, including the use of anti-lymphocyte globulin as a part of a tolerance induction regimen (Bas et al, 1992), the use of homozygous typing cells rather than the original donor cells in MLR (Reinsmoen et al, 1990, 1993) and the application of different methods to define DSH. Among living-related recipients, the reported incidence of DSH within the first two years post-transplant ranges from 95-100% for HLA-identical recipients and 31-47% in HLA-haploidentical grafts (Fletcher et al, 1984; Kahan et al, 1989; Kerman et al 1997).

The clinical relevance of DSH is that it has been shown to correlate with a favourable long-term graft outcome. Patients who show DSH have a lower incidence of acute rejection after the first three months post-transplant (Reinsmoen et al, 1990, 1993, 1994; Kerman, 1997; Creemers, 1997; Ghobrial, 1994) and a better 3-year (Reinsmoen et al, 1993) and 10-year (Kerman, 1997) graft survival. However, it should be noted that Steinmann et al (1994) showed no correlation between DSH and graft outcome, despite using a number of techniques to assess alloresponsiveness. Although DSH has also been found to be associated with a lower risk of chronic rejection in renal, heart and lung transplant recipients (Reinsmoen et al, 1994), other reports have shown that DSH may still occur in some patients with biopsy-proven chronic rejection (Reinsmoen et al, 1993; Bohmig et al, 2000). Therefore the detection of DSH may help to identify patients at low risk of

adverse immunological events, but its presence is not exclusive to patients with good graft outcome.

METHODS OF DETECTING DSH

Cellular assays have been developed to test for various effector and cytotoxic functions of T cells and have been used to assess the immune profile of solid organ recipients. One goal of these studies has been to determine if post-transplant changes in donor-specific cellular response could predict good versus poor graft outcome, which would allow individualisation of immunosuppression (Reinsmoen et al, 2002). Many techniques have been used to identify patients who show donor-specific hyporesponsiveness towards donor antigens. These include the mixed lymphocyte reaction, cell-mediated lympholysis, limited dilution assays, and most recently, in-vivo delayed-type hypersensitivity.

The mixed lymphocyte reaction (MLR), which measures the proliferative activity of recipient T cells against donor Class II MHC antigens, was the first technique used to demonstrate DSH (Amos and Bach, 1968). These investigators postulated that non-reactivity was caused by the absence of clones that can react to the allo-grafted HLA antigens and even at this early stage, Bach et al (1972) speculated that it might be possible to withdraw immunosuppression in recipients showing DSH. Since then, the MLR has been used widely to demonstrate antigen-specific hyporeactivity in vitro following renal transplantation (Charpentier, 1982; Fletchner et al, 1984; Kahan et al, 1989; Reinsmoen, 1990, 1991, 1993, 1994; Bas et al, 1992, 1993; Ghobrial et al, 1994; Fugiwara, 1994; Iniotaki-Theodoraki et al,

1995; Kim et al, 1996; Jackson et al, 1997; Kerman et al, 1997; Creemers et al, 1997; Salomao et al, 1998; Ishido et al, 1999; Bohmig et al, 2000). However, as noted above, considerable variability has been reported and the reliability of the assay is affected by a number of factors, including the source of the serum used in the culture medium, the nature and number of the stimulator cells, as well as the fact that patients have been selected according to widely varying criteria.

Another way that has been used to demonstrate DSH is the cell-mediated lympholysis (CML) assay which measures recipient cytotoxic T cell activity, mainly against donor Class I MHC antigens (Thomas et al, 1977; Goulmy et al, 1981; Pfeffer et al, 1983; Ono et al, 1983; Cohen et al, 1985; Fletchner et al, 1986; Wrammer et al, 1987; Fugiwara et al, 1994). The largest of such studies concluded that the correlation between CML responder status and graft outcome was of limited prognostic value (Goulmy et al, 1981). Although Fletchner et al (1986) reported a good correlation between MLR and CML assays, this was disputed by Fugiwara et al (1994) who found that persistent donor-specific responsiveness could often be detected by MLR, when there appeared to be unresponsiveness in the CML assay. As a result of these conflicting data, lack of correlation with graft outcome, and the fact that CML mainly measures Class I responses compared with Class II responses in MLR, the CML assay is now rarely used to assess donor-specific responsiveness.

Limited dilution assays (LDA) measure the frequency of precursors for cytotoxic T cells (pCTL) or IL-2 secreting peripheral blood T cells (pHTL) in the recipient following stimulation with donor cells and has also been used widely to demonstrate DSH (Bishop et al, 1989; Grailer et al, 1991; Hadley et al, 1992; Mason et al, 1996; Beik et al, 1997; Hornick et al, 1997; Van Besouw et al, 2000).

Although pCTL frequencies were reported to be useful in identifying kidney recipients in whom immunosuppression could be reduced (Beik et al, 1997; Van Besouw et al, 2000), others have reported low precursor frequencies may occur in a substantial number of recipients with chronic rejection (Mason et al, 1996; Hornick et al, 1998 and Baker et al, 2001). These discordant results may be the result of the relatively small number of recipients tested, different immunosuppressive regimens, varying LDA methods or the use of a single time point to define hyporeactivity rather than kinetic analysis of responder status (Reinsmoen, 2002). Additionally, pCTL is a measure of Class I responses and this assay is difficult to perform.

Since my work was carried out, in-vivo delayed-type hypersensitivity (DTH) assays have been used to assess the immune status of allograft recipients (Van Buskirk et al, 2000; Geissler et al, 2001). This method involves the injection of recipient peripheral blood mononuclear cells, together with donor antigen into the footpads or pinnae of naïve mice. If the recipient had been sensitised to donor antigens, a DTH-like swelling develops in the mouse skin due to recognition of the donor antigen and resultant inflammation. Geissler et al (2001) tested liver transplant recipients who had stopped all immunosuppression and reported that the DTH assay was better than the conventional CML and MLR assays for detecting donor antigen-linked suppression and suggested that the DTH assay may be a suitable method of assessing tolerance in clinical trials.

To date, only a few studies have measured T cell reactivity before and after the reduction of immunosuppression (Goulmy et al, 1991; Mazariegos et al, 1995; Beik et al, 1997; Creemers et al, 1998; Van Besouw et al, 2000, 2002), with one group showing that there was no rebound effect of increasing donor-specific

reactivity up to two years after tapering of immunosuppression (Van Besouw et al, 2002). However, only one centre has used immunoassays prospectively to reduce immunosuppression in patients who demonstrate DSH, but this was only attempted in living-related allograft recipients (Fletcher et al, 1984; Kahan et al, 1989; Kerman et al, 1997). MLR-defined DSH was detected in all HLA-identical recipients and in 47% of the haplo-identical recipients. These patients were selected for steroid withdrawal, but the five-year (Kahan et al, 1989) and the ten-year follow-up results (Kerman et al, 1997) showed variable success on cyclosporin monotherapy, with both acute rejection episodes and graft losses occurring after steroid withdrawal. Hence, there remains no sensitive or specific in vitro tests that reliably predict the success or failure of steroid withdrawal.

All in vitro assays have been subject to criticism and the method used to assess allo-reactivity appears to influence the percentage of recipients identified as being hyporesponsive. The combined literature indicates that DSH is identified in 70% of recipients by the CML assay and in only 30% of recipients by MLR (Reinsmoen, 2002). A method that underestimates the incidence of DSH is likely to be better than a method that over-estimates it, as the consequences of a false positive test could result in allograft rejection or graft loss. In my study I have used the MLR to assess proliferative responses, as MLR reactivity reflects MHC Class II disparity which has the biggest influence on acute rejection in clinical practice. In addition, MLR-defined DSH correlates with graft outcome, and this is the only technique which has been used to tailor immunosuppression.

DEFINITION OF DSH

As with the techniques used to detect DSH, there is also no consensus on how it should be defined and many investigators do not substantiate in any detail the definition chosen in their studies. In most cases, DSH has been defined by comparing pre-transplant proliferative responses against donor and third party (ThP) control cells, with the equivalent responses found after transplantation. Here, I will discuss only those definitions that have been applied to MLR-based assays, as this was the method I used to detect DSH.

Firstly, DSH has been defined using the relative response index (RRI), which measures the recipient's proliferative response to donor cells in proportion to that found against third party cells. It is calculated by the following formula:

$$\text{RRI (\%)} = \frac{\text{cpm (Recipient + Donor)} - (\text{Recipient} + \text{Recipient})}{\text{cpm (Recipient + Th P)} - (\text{Recipient} + \text{Recipient})} \times 100$$

where (Recipient + Recipient) represents the background response obtained using autologous stimulators. Some investigators have defined DSH as a two-thirds reduction in the RRI post-transplant compared with the pre-transplant RRI (Reinsmoen et al, 1990, 1993, 1994; Bas et al, 1992), while others have defined DSH as an RRI of $\leq 20\%$ at any time after transplantation (Colombe et al, 1989; Ghobrial, 1994; Kim, 1996; Salomao et al, 1998; Ishido, 1999). One group reported that a RRI of $\leq 20 \pm 8\%$ correlated with good graft outcome after eight years post-transplant (Salomao et al, 1998).

A second way of defining DSH has been to compare the stimulation indices (SI) obtained with donor stimulation and third party stimulation. The SI are calculated by the following formulae:

$$SI = \frac{cpm (\text{Recipient} + \text{Donor})}{cpm (\text{Recipient} + \text{Recipient})}$$

$$cpm (\text{Recipient} + \text{Recipient})$$

$$SI (\text{Th P}) = \frac{cpm (\text{Recipient} + \text{Th P})}{cpm (\text{Recipient} + \text{Recipient})}$$

$$cpm (\text{Recipient} + \text{Recipient})$$

Again, multiple cut-off values have been used to define DSH by this method. In some cases, patients who displayed a donor-specific SI < 6.5 and a third party SI > 6.5 were considered to show DSH (Fletcher et al, 1984; Kahan et al, 1989), whereas Kerman et al (1997), working in the same centre, used a donor-specific SI ≤ 10 and a third party SI > 10 to define DSH. In another centre, DSH was defined as a donor-specific SI < 6 with a third party SI > 6, and this correlated with good graft survival (Salomao et al, 1998). I did not select this method to define DSH as the definition of a meaningful SI appeared to be arbitrary.

Thirdly, DSH has been defined by the donor-specific reactivity (DSR), which calculates donor-specific proliferation as a proportion of the third party response obtained when a panel of three stimulator cells are used as the third party control (Creemers et al, 1997). It is calculated by the following formula:

$$DSR = \frac{(\text{Recipient} + \text{Donor})}{0.33 \times [\text{Recipient} + (\text{Panel of 3})]}$$

As this method does not take into account autologous background proliferation, which is an important factor in assessing the donor-specific proliferative response, it was not considered appropriate for my study.

Lastly, DSH has been defined as the “relative specific reactivity”, which represents donor-specific proliferation as a proportion of the recipient’s mean proliferative response against a panel of 5 individual third party control cells at each time-point post-transplant compared with the respective pre-transplant responses (Bohmig et al, 2000). It can be calculated by the following formulae:

$$\text{Donor-specific reactivity (\%)} = \frac{\text{cpm (Recipient + Donor) (post-Tx)}}{\text{cpm (Recipient + Donor) (pre-Tx)}} \times 100$$

$$\text{General reactivity (\%)} = \frac{\text{cpm } 0.2 \times [(\text{Recipient} + (\text{Panel of 5})) \text{ (post-Tx)}}]{\text{cpm } 0.2 \times [(\text{Recipient} + (\text{Panel of 5})) \text{ (pre-Tx)}}} \times 100$$

$$\text{Relative specific reactivity (\%)} = \frac{\text{Donor-specific reactivity}}{\text{General reactivity}} \times 100$$

This method was reported to minimise the effects of uraemia, infection or changes in immunosuppressive regimen and to give a more accurate representation of donor-specific T cell alloresponses as it took into account pre-transplant donor-specific and third party responses at each time point post-transplant. However, I did not use this method to define DSH, as pre-transplant MLR studies could not be performed on the retrospective cohort in my study.

The fact that many overlapping, but distinct and often arbitrary methods have been used to define DSH has undoubtedly contributed to the discrepancies in the predictive value of the phenomenon. I chose to define DSH using the RRI, as it the most widely used method, takes into account background proliferative responses, and allows for single time-point analysis which was necessary in the retrospective cohort of my study.

I also assessed donor-specific cytokine production, as MLR-defined DSH on its own, has not proven to be sufficient in guiding reduction of immunosuppression. The underlying mechanism of DSH remains unknown, but as I have discussed above, it may involve active immune regulation. The possibility that DSH is associated with the production of inhibitory cytokines in response to donor stimulation has only been investigated in case reports of living-related recipients who showed DSH while

maintained without immunosuppression (Kusaka et al, 1995; Christensen et al, 1998; Ishido et al, 1999). These reports suggest that DSH may be associated with a Th2 cytokine profile. As most transplant patients are maintained on immunosuppression, it would be important to determine whether the assessment of donor-specific cytokine production is of value under these circumstances.

SUMMARY AND AIMS

Transplantation has become the preferred mode of renal replacement therapy for most patients with end-staged renal failure. However, the induction of specific immunological tolerance is not yet possible and immunosuppressive therapy remains the mainstay of treatment. New immunosuppressive agents have further improved short-term allograft survival, but some have been in clinical use for too short a period to determine their effect on long-term graft survival. Unfortunately, some of these agents also increase the risk of infection, malignancy and heart disease. Theoretically, minimisation or elimination of the side effects of immunosuppressive drugs should be possible by substitution of toxic drugs, or by the withdrawal of a specific drug in stable patients. However, clinical trials have shown varied outcomes of doing this and until there is a reliable immunological method for selecting appropriate patients for reduction of immunosuppression, it is likely that many will receive more immunosuppression than is actually needed to protect the graft.

The study of allograft tolerance is complicated by the use of immunosuppression in clinical transplantation and true antigen-specific tolerance has been described only in case reports and in small series of patients who have

discontinued immunosuppression. DSH appears to represent a tolerance-like state in immunosuppressed individuals which may be associated with better long-term graft survival due to a reduced incidence of acute and chronic rejection. Although the mechanism of DSH remains unknown, it may involve one or more of the mechanisms implicated in other forms of peripheral T cell tolerance, including clonal anergy, clonal deletion, and cytokines produced by regulatory T cells. Previous investigators have used a number of different techniques to demonstrate DSH, but the use of multiple different definitions to define DSH makes it difficult to interpret many of these studies.

The aim of my study was to determine whether good graft outcome correlated with the presence of DSH and the selective production of inhibitory cytokines that have been associated with the induction of antigen-specific tolerance. DSH was detected by MLR and cytokines were analysed by ELISA and RT-PCR. The standardisation of these techniques is described in Chapter 2. The results of a group of long-term surviving cadaveric recipients with varied allograft outcome, referred to as the Cadaveric Cohort, are described in Chapter 3, while a group of living-related recipients, the Living-related Cohort, is described in Chapter 4. A small group of cadaveric recipients on low-dose immunosuppression as a result of solid-organ malignancy, the Malignancy Cohort is discussed in Chapter 5. Chapter 6 then outlines the evolution of DSH and sequential cytokine analysis in a group of cadaveric and living-related recipients during the first year post-transplant, the Prospective Cohort. Finally, Chapter 7 is a general discussion of my findings in relation to previous work and highlights areas of potential future study.

CHAPTER 2: MATERIALS AND METHODS

STUDY DESIGN

This study was performed in two arms. The retrospective study examined a cohort of cadaveric and living related recipients with long-term functioning renal allografts, and provided a foundation for interpretation of the prospective study. The retrospective arm of the study was designed to determine whether good allograft outcome in long-term graft survivors was associated with donor-specific hyporesponsiveness and a specific pattern of cytokine production. The prospective study was carried out to determine whether patients who developed DSH within the first year post-transplantation had a similar cytokine profile to that of long-term survivors who show DSH. The overall aim was to test whether cytokines might be of predictive value in tailoring immunosuppression.

Approval was obtained from the local Ethical Committee before patients were recruited. The proposal outlined the main aims of the project, materials and methods (including the use of radioactive substances and information regarding certification for laboratory use and disposal), procedures involving patients and volunteers, safety and funding. Patients were asked to participate in the study at a routine clinic visit. An information sheet about the project, which was also approved by the Ethical Committee, was given to all patients and 30ml blood was then obtained after informed consent.

Retrospective Study

Patients considered for this study had to be first renal allograft recipients, excluding all those who were pregnant and unable to give informed consent. A database search was performed to identify suitable patients. Recipient details, including date of birth, date of transplant, HLA type, panel reactive antibody (PRA) status, baseline and current immunosuppressive regimes, and level of graft function were obtained from computer and casenote records. The PRA represents the recipient's level of sensitisation against a panel of known Class I and II HLA antigens prior to transplantation and was measured routinely at three-monthly intervals while on the transplant waiting list.

An extensive casenote search was then carried out to verify acute and chronic rejection history based on histopathology reports. As there was no reliable record of blood transfusion prior to transplantation in the casenotes, each participant was asked about previous blood transfusions at the time of giving consent. In all cases, these had been given as treatment for anaemia and not as part of a planned pre-transplant blood transfusion programme. T-cell complement dependent cytotoxicity cross match was also negative prior to transplantation in all patients.

Donor information was obtained from the Tissue Typing Laboratory at Glasgow Royal Infirmary. The tissue type and degree of HLA-mismatch to the recipient were obtained from laboratory records for all patients. Donor cells were extracted from spleen following organ retrieval, cryopreserved and the number of cryopreserved cells recorded. Patients identified from the database search were considered for the study only if adequate donor material was available. Patients were

then entered into the study in one of three cohorts based on the criteria described below. Clinical details of patients are given in the appropriate chapters.

CADAVERIC COHORT

All patients were first renal allograft recipients with a functioning transplant for more than five years. Two patient groups were defined to determine whether the presence of donor-specific hyporesponsiveness correlated with graft outcome.

GROUP A

Patients in this group had a good allograft outcome, as shown by no acute rejection beyond the first three months post-transplant and good allograft function with a serum creatinine $< 140 \text{ mol/l}$. There was no history of chronic rejection.

GROUP B

Patients in this group had poor allograft outcome with either late, multiple or steroid-resistant acute rejection, or biopsy-proven chronic rejection. Their level of graft function was not defined biochemically.

LIVING-RELATED COHORT

This cohort comprised recipients with a functioning renal allograft from a living-related donor. The number of patients with a functioning graft for more than five years whose donor was available to provide cells was small. Therefore, patients with a functioning primary allograft for more than three years were considered. They were analysed in the same outcome groups defined above in the cadaveric cohort.

Once consent was obtained from the recipient, the organ donors were contacted by telephone or letter to request their participation. Clinical information on each recipient and donor was obtained from computer and casenote records. Details of HLA-typing and level of sensitisation were obtained from the Tissue Typing Laboratory.

MALIGNANCY COHORT

The final cohort included patients who had developed a histologically confirmed, solid organ malignancy post-transplant. These patients were studied as they have maintained their grafts despite minimal immunosuppression. All were first graft recipients and had a functioning allograft for more than five years, but the level of graft function and rejection history were not used as selection criteria. The type of malignancy, organ involvement and time of diagnosis post-transplantation are described in Chapter 5.

Prospective Study

Recipients of first cadaveric or live-donor renal allografts were eligible for this arm of the study. Again, the only exclusion criteria were pregnancy and inability to give informed consent. The follow-up period was one year and blood was obtained from each patient immediately before transplantation, and then at 3 months, 6 months and 12 months post-transplantation. Donor cells were cryopreserved in the Tissue Typing laboratory at the time of organ retrieval and stored. Recipient information

including the age, tissue type, and previous level of sensitisation was obtained from computer and casenote records. The clinical course of each patient was followed, with a record kept of the immunosuppressive regime, level of graft function, rejection history and graft loss. In order to reduce variability, all the assays of cellular function and cytokine production were performed in parallel on completion of the follow-up period when all samples had been collected. Samples of some patients who lost their grafts before the end of the study period were also analysed.

CELL ISOLATION

Recipient (Responder) Cells

In the Retrospective Arm of the study, blood was obtained prior to the morning dose of cyclosporin or tacrolimus at a routine clinic visit. In the Prospective Arm of the study, blood was obtained immediately before transplantation and thereafter at routine clinic visits at 3, 6 and 12 months post-transplant. Peripheral blood mononuclear cells (PBMC) were isolated from 30 ml blood in EDTA using standard density gradient centrifugation (Lymphoprep, Nycomed, Norway). 10 ml whole blood was layered carefully onto 15 ml Lymphoprep in a 50 ml centrifuge tube. This was centrifuged for 30 minutes at 1500 rpm and then the cell layer was harvested, washed twice in RPMI 1640 (GibcoBRL, Paisley, UK) and the viability and cell count assessed by phase contrast microscopy. The cells were suspended in foetal calf serum supplemented with 10% DMSO, gradually frozen to -70°C and cryopreserved in liquid nitrogen until ready for use.

Third party stimulator cells

As it was not feasible to obtain a single third party control with the same degree of HLA-mismatch as the recipient-donor pair for all of the patients in this study, a pool of stimulator cells from 5 unrelated normal volunteers was used. The tissue types of the members of the pool, representing seven different HLA-DR types (DR 1, 2, 3, 4, 6, 7, 8), are shown in Table 2.1. Cells from each pool member were obtained using the method described above and cryopreserved individually. To create the third party pool control, one aliquot from each member was thawed and equal numbers of each were mixed. All allograft recipients in this study were tested against the same pool.

Table 2.1: HLA-types of the pool members used in the MLR for third party selection.

POOL MEMBER	HLA - A	HLA - B	HLA - DR
1	1, 8	27	3, 2/15
2	2, 3	13, 27	1, 4
3	1, 2	5, 8	3/17, 8
4	1, 11	8, 17	4, 6/13
5	1	57	7

Donor splenocytes

Donor spleen tissue was obtained at the time of organ retrieval and the cells were extracted and cryopreserved by the Tissue Typing Laboratory at Glasgow Royal Infirmary. The cells were transported in liquid nitrogen to the Western Infirmary for immediate storage in liquid nitrogen.

MIXED LYMPHOCYTE REACTION

Standardisation of Protocol

The MLR method described below was devised on the basis of optimal conditions which were established by the following experimentation. First, foetal calf serum (FCS) was compared with human AB serum (from untransfused male donors) as a supplement in the culture medium (Figure 2.1). Secondly, a range of responder and stimulator cell numbers was tested. The highest donor-specific proliferation was achieved with a cell number of 2×10^5 cells/well (Figure 2.2a) and the highest proliferation to mitogenic stimulation was achieved with a responder cell number of 1×10^5 cells/well (Figure 2.2b). Lastly, a time course study was performed to determine the optimal duration of culture. Peak proliferation following stimulation with 1mg/ml PHA (Murex Diagnostics, Temple Hill, Dartford, UK) was consistently shown to be on Day 3 (Figure 2.3a) and on Day 6 for unrelated stimulator cells (Figure 2.3b).

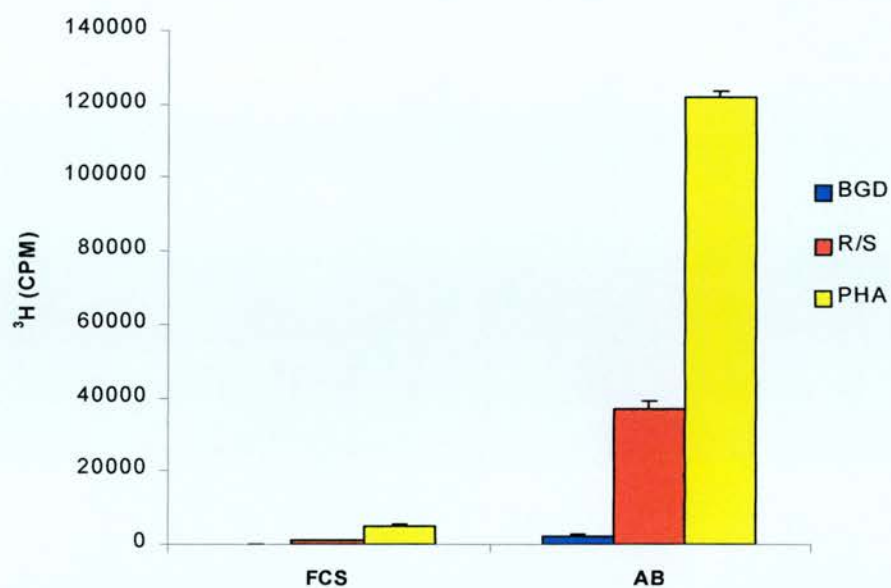


Figure 2.1: Comparison of MLR responses in the presence of FCS or human AB serum. Results shown are mean ^3H -TdR incorporation ± 1 s.d. for triplicate cultures of normal volunteer responder cells stimulated for 6 days with cells from an unrelated volunteer (R/S) or for 3 days with 1mg/ml PHA in medium containing 10% FCS or AB serum. BGD represents the background responses obtained from responder cell stimulated with autologous stimulators.

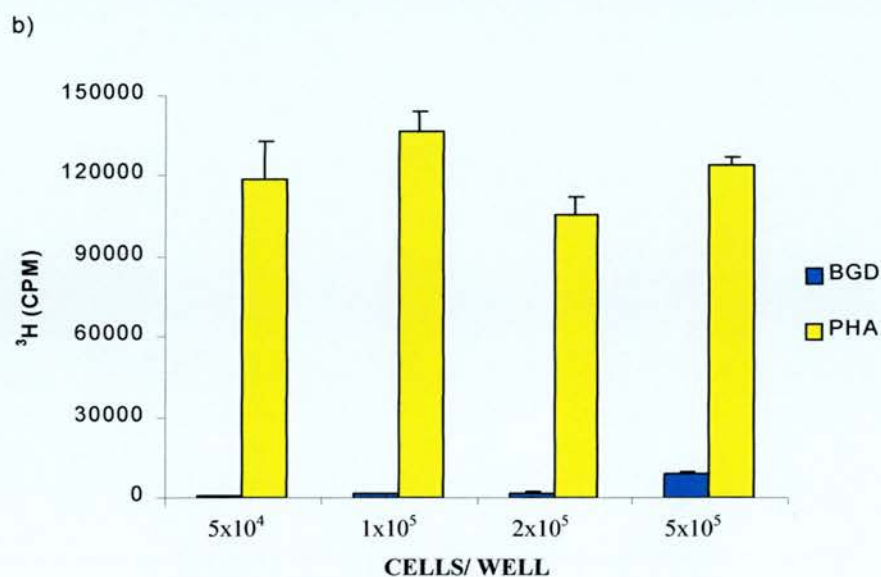
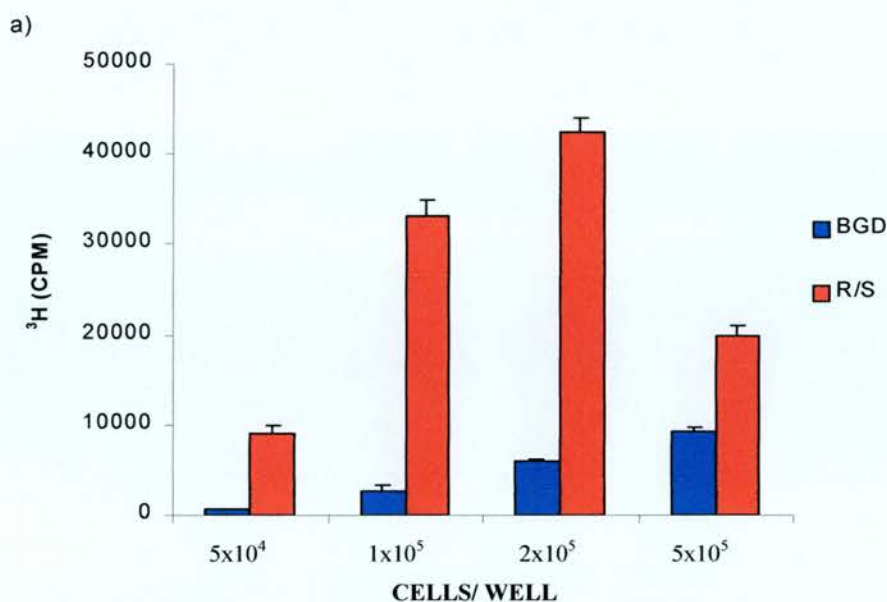


Figure 2.2: Dependence of MLR responses on donor cell numbers. Results shown are mean ^3H -TdR incorporation \pm 1 s.d. of different numbers of normal responder cells cultured in the presence of similar number of stimulator cells from an unrelated volunteer (R/S) for 6 days (a) or 1mg/ml PHA (b). BGD represents the background responses obtained from responder cell stimulated with autologous stimulators.

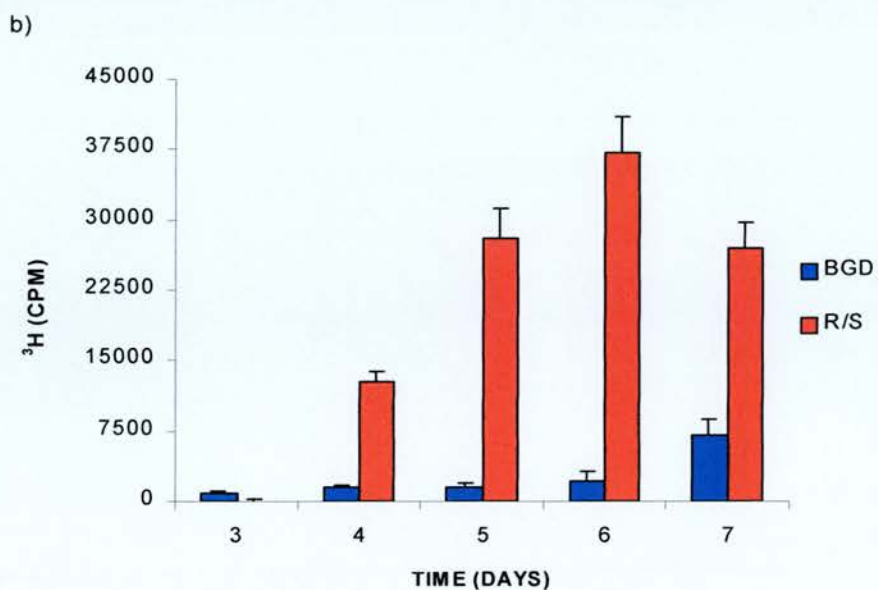
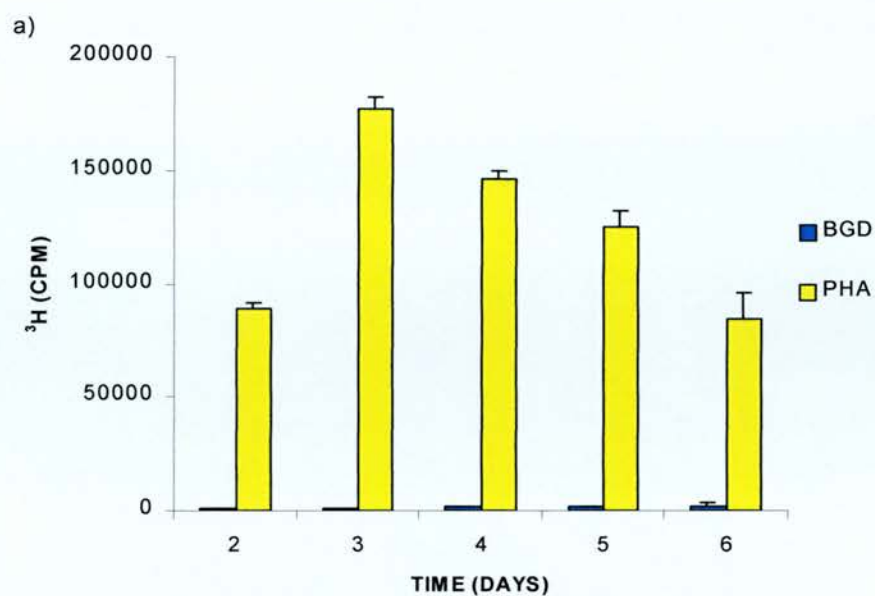


Figure 2.3: Time course study of mixed lymphocyte responses. Results shown are mean ^3H -TdR incorporation \pm 1 s.d. of normal responder cells ($2 \times 10^6/\text{ml}$) cultured in the presence of 1mg/ml PHA (a) or stimulator cells ($2 \times 10^6/\text{ml}$) from an unrelated volunteer (R/S) for 6 days (b). BGD represents the background responses obtained from responder cell stimulated with autologous stimulators.

MLR METHOD

Donor and recipient cells were thawed rapidly, washed in RPMI 1640 (GibcoBRL, Paisley, UK) supplemented with 50% human AB serum (Quest Biomedical, Knowle, West Midlands, UK) and assessed for viability by phase-contrast microscopy. Donor, third party and autologous stimulator cells were treated with 50µg/10⁷ cells/ml mitomycin c (Sigma, Poole, Dorset, UK) for 45 minutes at 37°C, washed three times, and brought to a working concentration of 2 x 10⁶ cells/ml. Equal numbers of responder and mitomycin c treated stimulator cells at 2 x 10⁶ cells/ml were cultured in RPMI 1640 supplemented with 10% human male AB serum, 2mM L-glutamine (GibcoBRL), 100µg/ml penicillin (GibcoBRL) and 100µg/ml streptomycin (GibcoBRL) in 96 well round-bottomed tissue culture plates (Corning Costar, High Wycombe, Bucks, UK) for 6 days at 37°C in a humidified atmosphere of 5% CO₂ in air. As a positive control, responder cells were also cultured with 1mg/ml PHA (Murex Diagnostics, Temple Hill, Dartford, UK) for 3 days. Proliferation was assessed by adding 1 µCi/well ³H-thymidine (West of Scotland Radionuclide Dispensary) during the final 18 hours of incubation and DNA-bound ³H-thymidine was harvested using a Betaplate cell harvester (Wallac).

DSH has been defined by the relative response index (RRI) which is calculated by the following formula:

$$\text{RRI} = \frac{(\text{Recipient} + \text{Donor}) - (\text{Recipient} + \text{Recipient})}{(\text{Recipient} + \text{Third Party}) - (\text{Recipient} + \text{Recipient})} \times 100$$

where (Recipient + Recipient) represents the autologous background. Initially, I used an RRI of ≤ 20% as a definition of DSH (Ghobrial 1994).

MEASUREMENT OF CYTOKINE LEVELS BY ELISA

IL-2, IL-4, IL-10 and IFN- γ

A sandwich ELISA was developed to measure IL-2, IL-4, IL-10 and IFN- γ using matched antibody pairs (R&D Systems, Abingdon, Oxon, UK). The optimal concentrations of capture antibody, detection antibody, and standard were determined using checkerboard protocols. Supernatants were harvested from MLR cultures, centrifuged at 13,000g and stored at -20°C before use. 96-well flat-bottomed plates (Nunc-Immuno) were coated with capture antibody in PBS (pH 7.2) at a concentration of 4 $\mu\text{g/ml}$ and incubated overnight at 4°C . The plates were then blocked with PBS/ 1% bovine serum albumin (Sigma) for 2 hours at 37°C . 50 μl culture supernatant in triplicate, and serial dilutions of recombinant human IL-2, IL-4, IL-10 and IFN- γ (R&D Systems) as standards in duplicate, were added to plates and incubated overnight at 4°C .

The plates were then washed with PBS/ 0.05% Tween 20 and detecting antibody (anti-IL-2 & IL-4: 12.5 ng/ml; anti-IL-10: 500 ng/ml; anti-IFN- γ : 200 ng/ml) was added for 3 hours at 37°C . Extravidin peroxidase (Sigma) was added for 2 hours at 37°C followed by TMB substrate (KPL, Maryland, USA) for approximately 20 minutes and the plates were read at 630 nm with a reference wavelength of 405nm using an automated plate reader (Dynex Technologies). The concentration of cytokine was calculated using standard curves derived from the recombinant cytokines and the levels obtained using autologous stimulator cells were subtracted to obtain donor-specific cytokine production.

A time course study was then performed to determine the optimal day for harvesting supernatants of the MLR. Cells from normal individuals were used as responders and stimulators in the MLR, PHA was used as a positive control and supernatants were harvested on Days 1 to 4. The secretion of IL-2, IL-4, IL-10 and IFN- γ was quantified on each day of the time course (Figure 2.4). The peak cytokine production with stimulator cells was on Day 3 for all cytokines measured. The optimal day for harvesting of supernatants stimulated with PHA was Day 1 for IL-4, Day 2 for IL-2 and IFN- γ , and Day 3 for IL-10. As cell numbers were limited, supernatants from PHA stimulated cells were harvested on Day 2 for all cytokines and from stimulator cells on Day 3 for all cytokines.

TGF- β

Activated TGF- β 1 was quantified using a commercial 'Duo Set' kit (R&D Systems, Abingdon, Oxon, UK). The block buffer consisted of 5% Tween 20, 5% Sucrose in PBS with 0.05% NaN₃. The diluent used for diluting standard, samples and detection antibody, consisted of PBS (pH 7.3) containing 1.4% delipidized bovine serum (R&D Systems, catalog #DY997) and 0.05% Tween 20.

Culture Conditions

Initially, I analysed TGF- β levels in supernatants of cultures performed in human AB serum, as I did for other cytokines. However, as shown in Figure 2.5a, the presence of serum produced very high background levels of TGF- β , obscuring any differences between samples. I then attempted to culture the cells in the absence of

serum, but this was also associated with background problems as shown in Figure 2.5b. For this reason, I eventually chose a method in which the cells were cultured in AB serum for 24 hours, before transferring to serum-free conditions. As shown in Figure 2.5c, this allowed more accurate differentiation of TGF- β production in the background (autologous and culture medium) and test samples. A time course study showed that the optimal day for harvesting of supernatants was Day 3 (Figures 2.5a-c).

ELISA Method

A 96-well flat-bottomed plate (Nunc-Immuno) was coated with capture antibody (2 $\mu\text{g/ml}$) diluted in PBS and incubated overnight at 4°C. The plate was washed with 0.05% Tween 20 in PBS and blocked for 2 hours with block buffer. All samples were activated prior to analysis by the addition of 25 μl 1N HCl to 125 μl cell culture supernatant for 10 minutes at room temperature. The sample was then neutralised by adding 25 μl 1.2 N NaOH/ 0.5 M Hepes and mixed well. Samples were diluted 2-fold by adding 75 μl of reagent diluent. A standard curve was constructed using recombinant cytokine (2 ng/ml) in doubling dilutions and 50 μl activated samples was added in triplicate and incubated overnight at 4°C. Detecting antibody (300 ng/ml) was added for a 3-hour incubation, before extravidin peroxidase in a concentration of 1:1000 (Sigma) was added for 2 hours at 37°C followed by TMB substrate (KPL). The levels of cytokine were analysed using a plate reader as described above.

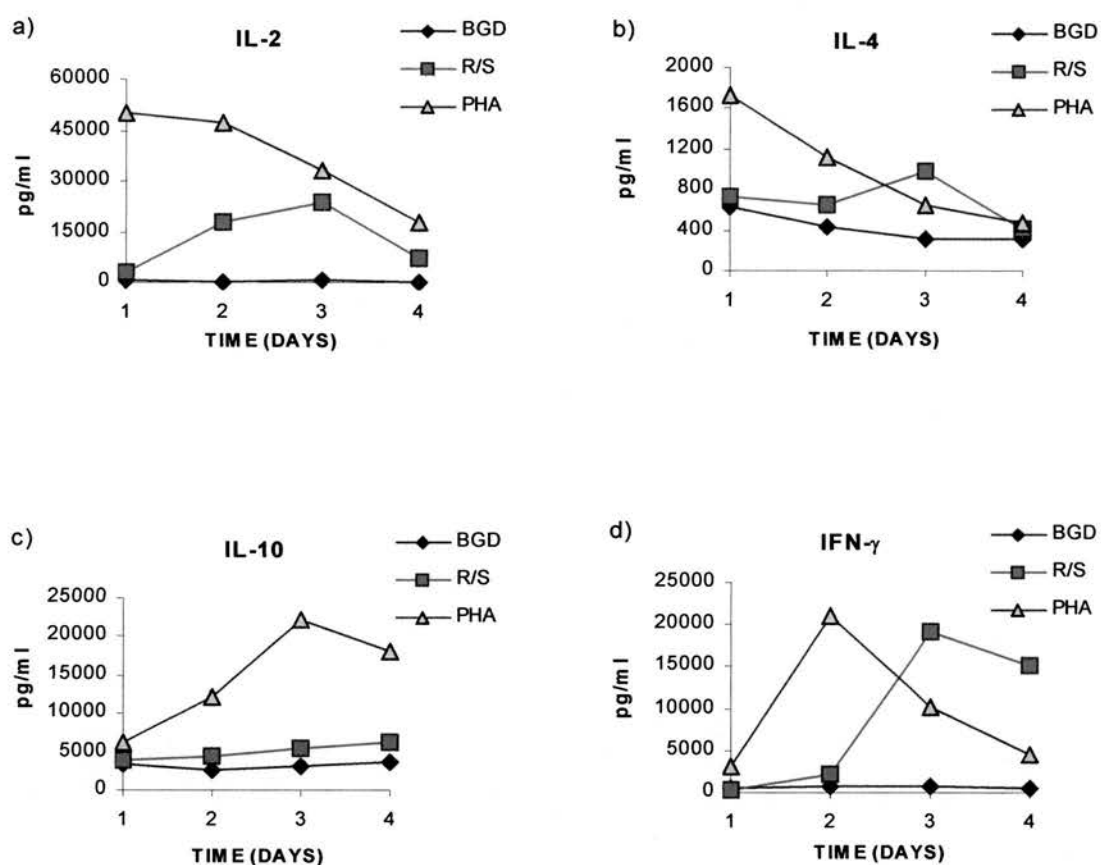


Figure 2.4: Time course of cytokine production during MLR and in response to PHA. Results shown are mean levels of IL-2 (a), IL-4 (b), IL-10 (c) and IFN- γ (d) in pg/ml measured by ELISA after 1-4 days of culture of cells from a normal volunteer stimulated with an unrelated volunteer (R/S) or 1 mg/ml PHA. BGD represents the background responses obtained from responder cell stimulated with autologous stimulators.

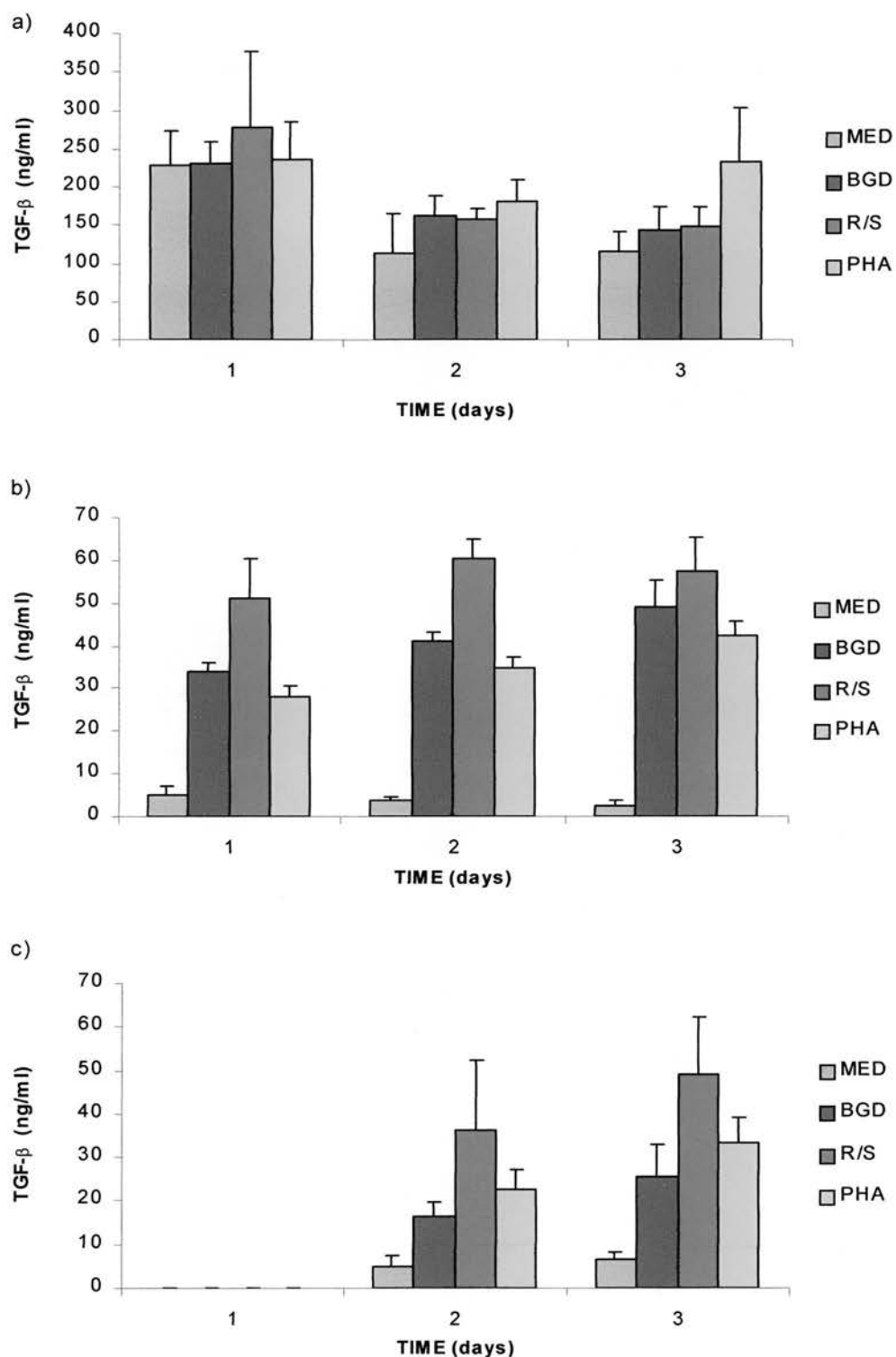


Figure 2.5: Time course of TGF- β production during MLR and in response to PHA. Results shown are mean levels of TGF- β in ng/ml \pm 1 s.d. measured by ELISA after 1-3 days of culture of cells from a normal volunteer in 10% AB serum (a), serum free conditions (b) or AB serum for the first 24 hours (c), stimulated with cells from an unrelated volunteer (R/S) or 1 mg/ml PHA. MED represents the response obtained in culture medium and BGD represents the background responses obtained from responder cell stimulated with autologous stimulators.

DETECTION OF mRNA EXPRESSION BY RT-PCR

ISOLATION of RNA

Cells were pelleted at 13 000g, washed in PBS at 4°C for 5 minutes, resuspended in 400 µl RNAzol™ B (Biogenesis Ltd, Poole, UK) and frozen at -70°C until ready for use. In preparation for RNA extraction, the cells were thawed and 40 µl chloroform was added. The sample was mixed vigorously for approximately 15 seconds, incubated on ice for at least 5 minutes and the resulting suspension centrifuged at 13 000g for 15 minutes at 4°C. 200 µl of the upper clear phase was carefully removed into a fresh RNase-free Eppendorf to which 200 µl isopropanol was added and stored on ice for 30 minutes or at 4°C overnight to allow the RNA to precipitate. The lower blue phenol-chloroform phase was discarded and the sample centrifuged at 13 000g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was washed in 500 µl 75% ethanol for 5 minutes at 4°C. The pellet was allowed to air dry before 10 µl sterile water was added and the pellet dissolved by heating to 68°C in a dehybridiser for 2 minutes. The optical density was read and the quantity of RNA was calculated (40 µg/ml RNA = A260 of 1.000).

REVERSE TRANSCRIPTION

The volume of RNA needed for 2µg RNA and the volume of water needed to make up a total volume of 10µl were calculated and the solution incubated in an Eppendorf with 2µl random primers (GibcoBRL) for 10 minutes at 70°C and then cooled on ice for at least 1minute. The RT mix was prepared using 2 µl 10x RT buffer (GibcoBRL), 2 µl 25mM MgCl₂ (GibcoBRL), 1 µl 10 mM dNTP (GibcoBRL), and 2

µl 0.1M DTT (GibcoBRL) for each reaction. 7µl RT Mix was added to each RNA/primer mixture, vortexed gently and centrifuged at 6000 rpm for a few seconds. The samples were then incubated for 5 minutes at 25°C before 1 µl (200 units) Superscript II RT (GibcoBRL, Paisley, UK) was added to each reaction, which were incubated for a further 10 minutes at 25°C, followed by 50 minutes at 42°C. The reaction was stopped by incubating for 15 minutes at 70°C and cooled on ice.

PCR

The PCR for each cytokine was optimised by investigating different magnesium concentrations and annealing temperatures for the primers listed in Table 2.2. All primers worked well at a [MgCl₂] of 1.5 mM and an annealing temperature of 60°C. A PCR Mix was prepared, consisting of 5µl 10X PCR buffer (GibcoBRL), 3µl 25mM MgCl₂ (GibcoBRL), 1µl 10mM dNTP (GibcoBRL), 2.5µl of each primer (40ng/ml, Sigma-Genosys, Cambridgeshire, UK) and 33µl distilled water for each reaction. 47µl of PCR Mix was aliquoted into pre-labelled PCR tubes and 2µl of cDNA was added to each tube, mixed and incubated for 2 minutes at 94°C in a DNA thermocycler (Jenson, PLS, East Sussex, UK). The program was paused, 1µl Taq DNA polymerase (GibcoBRL) was added, and the mixture was allowed to incubate for a further 3 minutes. 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute were followed by 72°C for 5 minutes and finally cooled at 4°C for a maximum of 99 hours. The samples and a 50 base pair DNA ladder (GibcoBRL) were analysed on 2% agarose gels (Gibco BRL) to which 20 µl ethidium bromide (Sigma) was added. The gels were run on a electrophoresis power supply (BioRad,

Hemel Hempstead, Herts, UK) using a UV transilluminator (Bio-Vision, Bio/Gene LTd, Kimbolton, Cambs, UK).

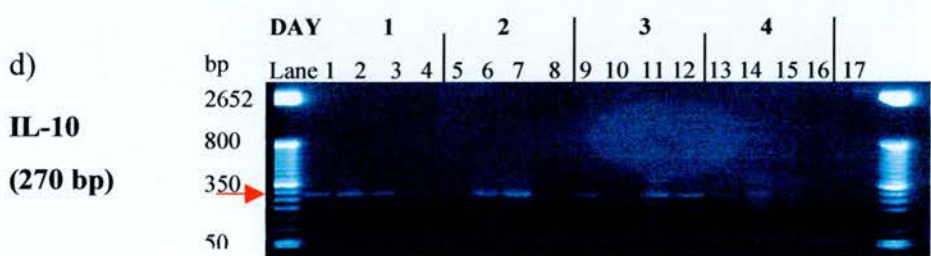
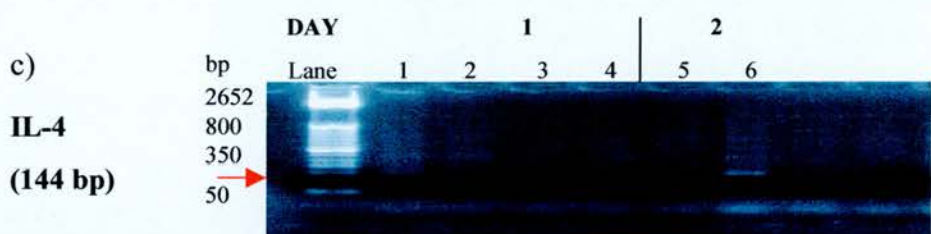
Following optimisation of the assay, a time course study was carried out using cells from volunteers to determine the optimal day for detecting IL-2, IL-4, IL-10, IFN- γ , and TGF- β gene expression. The house-keeping gene, β -actin, was detected in all samples as shown in Figure 2.6a. In PHA stimulated cells, mRNA for IL-2 (Figure 2.6b), IL-10 (Figure 2.6d), IFN- γ (Figure 2.6e), and TGF- β (Figure 2.6f) could all be detected from Days 1-3. Gene expression in the allo-MLR could be detected on Days 1 and 2 for IL-2 and IL-10, Day 2 only for IFN- γ and throughout the time course for TGF- β . IL-4 was very difficult to detect, but a weak band was seen in the allo-MLR on Day 2 (Figure 2.6c).



Table 2.2: Primer sequences and product sizes (base pairs) for human β -actin, IL-2, IL-4, IL-10, IFN- γ and TGF- β .

OLIGO NAME	SENSE	ANTI-SENSE	BASE PAIRS
HUMAN β -ACTIN	GGG GTA TGC CCT CCC CCA TGC CAT CCT GCG	TTG GCG TAC AGG TCT TTG CCG ATG TCC ACG	387
HUMAN IL-2	GCA CTA AGT CTT GCA CTT GTC ACA AAC	TAC AAT GGT TGC TGT CTC ATC AGC ATA	375
HUMAN IL-4	ATG AGA AGG ACA CTC GCT GC	TCT GGT TGG CTT CCT TCA CAG	144
HUMAN IL-10	ATG CTT CGA GAT CTC CGA GA	AAA TCG ATG ACA GCG CCG TA	270
HUMAN IFN- γ	CAG ATG TAG CGG ATA ATG G	GCA GGA CAA CCA TTA CTG G	388
HUMAN TGF- β	CAG AAA TAC AGC AAC AAT TCC TGG	TTG CAG TGT GTT ATC CGT GCT GTC	188

IL-2: Interleukin-2, IL-4: Interleukin-4, IL-10: Interleukin-10, IFN- γ : Interferon-gamma, TGF- β : Transforming growth factor-beta.



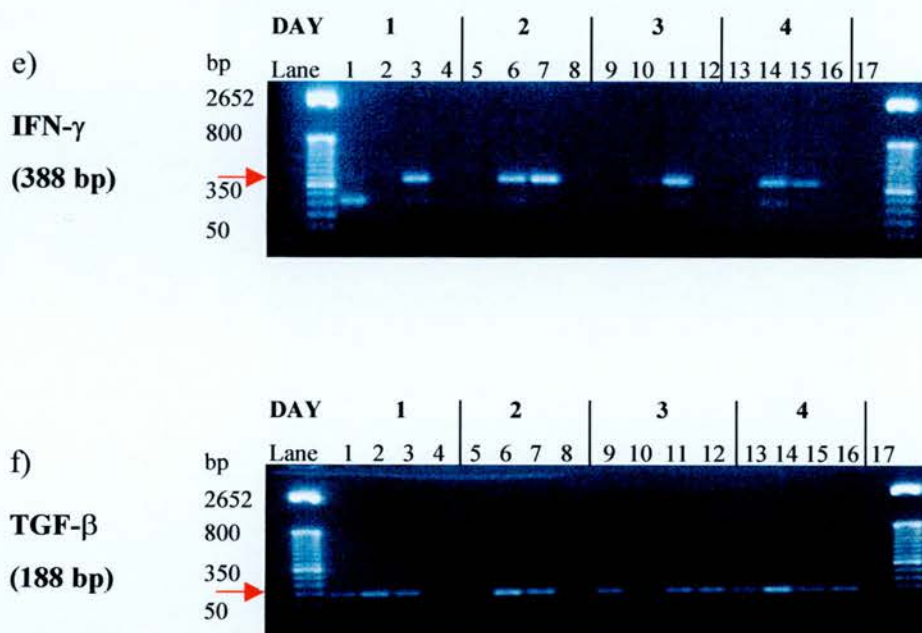


Figure 2.6: Time course of cytokine mRNA expression in MLR or in response to PHA. β -actin (a), IL-2 (b), IL-4(c), IL-10 (d), IFN- γ (e), TGF- β (f) mRNA were assessed by PCR after culture for 1-4 days (with autologous stimulators (BGD - lanes 1, 5, 9, 13), allogenic stimulator cells (lanes 2, 6, 10, 14), PHA (lanes 3, 7, 11, 15) and in stimulators cells only (lanes 4, 8, 12, 16). Negative control wells contained no mRNA (lane 17).

STATISTICAL ANALYSIS

Clinical parameters (age, duration since transplant and serum creatinine) have been expressed as medians and ranges for sub-groups of patients and were compared using the Wilcoxon signed rank test, with $p < 0.05$ being considered significant. Cytokine results were expressed as means and standard error of the means and were also compared using the Wilcoxon signed rank test. Linear correlation was used to determine the relationship between RRI and serum creatinine and cytokine production. A receiver operating characteristic (ROC) curve was used to construct a graphic representation of the relation between the sensitivity and specificity of the laboratory test used to detect DSH over all possible diagnostic cut-off values (Altman, 1994). The sensitivity and specificity of each observed data value was calculated and the ROC curve was constructed by plotting sensitivity against 1-specificity. The positive and negative predictive values of each potential cut-off of RRI were also determined.

CHAPTER 3: CADAVERIC COHORT

INTRODUCTION

At present, there remains no reliable method of identifying renal transplant patients who are suitable for reduction of immunosuppressive therapy and the only strategy is vigilance and early treatment of complications. The main aim of the Retrospective Arm of the study was to determine whether donor-specific hyporesponsiveness correlated with long term allograft outcome and the production of inhibitory cytokines. This chapter describes the cadaveric cohort, which forms the main part of this arm of the study. All the patients in this group had been transplanted for more than five years and many had experienced adverse effects from long term immunosuppression. Patients were divided into two groups based on allograft outcome, with the hypothesis that patients with good allograft outcome were more likely to show DSH and to produce inhibitory cytokines such as IL-4 and IL-10 compared with patients with poor graft outcome. Donor-specific hyporesponsiveness (DSH) was identified by proliferation in mixed lymphocyte cultures, cytokine secretion in MLR was measured by ELISA, and cytokine gene expression was determined in selected patients by RT-PCR.

PATIENTS

Thirty-six adult recipients (21 male and 15 female) of cadaveric renal transplants performed between February 1983 and March 1993 were studied. The inclusion criteria were first graft recipients who had been transplanted for more than 5 years.

The exclusion criteria were pregnancy and inability to give informed consent. The aetiology of renal failure was: chronic glomerulonephritis (n=10), chronic pyelonephritis (n=5), polycystic kidney disease (n=4), diabetic nephropathy (n=4), hypertensive nephrosclerosis (n=2), congenital renal dysplasia (n=2), Alport's syndrome (n=1), nephrocalcinosis (n=2), obstructive uropathy (n=1), post-partum renal failure (n=1), and unknown (n=4). Most patients received one or more blood transfusion during the course of renal replacement therapy prior to transplantation, as treatment for anaemia and not as part of a planned pre-transplant blood transfusion programme.

Classification of Patients

Patients were divided into two groups based on their history of acute and chronic rejection and level of graft function, as outlined in Chapter 2. Group A comprised 10 male and 8 females and Group B comprised 11 male and 7 female patients. The median age, time interval since transplant, degree of HLA-mismatch, serum creatinine and rejection history of both groups are shown in Table 3.1.

Rejection History

Acute rejection was diagnosed on the basis of deterioration in graft function and confirmed by percutaneous renal biopsy. Acute rejection episodes were treated with high dose steroids and one patient had a steroid-resistant episode, which required treatment with the monoclonal antibody OKT3. Chronic rejection was characterised by a progressive decline in graft function with proteinuria and was confirmed by percutaneous renal biopsy.

Table 3.1: Clinical features of cadaveric recipients in Groups A and B.

GROUP	AGE TX (years)	TIME TX (years)	M/M	PRA (%)	CREAT (μ mol/L)	EARLY AR		LATE AR	MULT AR	CR	GRAFT LOSS
						S-S	S-R				
A (n=18)	41.1 (11.3 - 57.8)*	10.3 (7.6 - 17.5)	3 (0 - 5)	4 (0 - 41)	102 (56 - 132)	4	0	0	0	0	0
B (n=18)	34.6 (19.3 - 62)	9.6 (7.5 - 15.7)	3 (0 - 5)	0 (0 - 88)	252 (160 - 480)	3	1	5	3	15	5

AGE TX: age at transplant, TIME TX: duration since transplant, M/M: degree of HLA-A, B and DR-mismatch. PRA: panel reactive antibody level, CREAT: creatinine. AR: acute rejection, MULTI: multiple, S-S: steroid sensitive, S-R: steroid resistant, CR: chronic rejection.

*Results are shown as medians and ranges where appropriate.

Immunosuppression

The baseline immunosuppressive regimens used in both groups are shown in Table 3.2. Fourteen patients in Group A and eleven patients in Group B received dual therapy (prednisolone combined with azathioprine or cyclosporin) and four patients in Group A and seven patients in Group B received conventional triple therapy (prednisolone, azathioprine, and cyclosporin). There were no changes in the regimen throughout the course of transplant follow-up in patients in Group A. However, the regimen for some patients in Group B was modified before or during the course of the study period due to late (after the first three months) or multiple acute rejection episodes and the development of chronic rejection (Table 3.2).

CLINICAL FEATURES OF CADAVERIC COHORT

There was no significant difference between patients in Group A and B with respect to age, time since transplant or degree of HLA-mismatch. The degree of sensitisation to HLA-antigens as measured by the panel reactive antibody (PRA) level was also similar in the two patient groups (Table 3.1). As expected, patients in Group A had a significantly lower median serum creatinine levels (102 $\mu\text{mol/L}$, range 56-132) than patients in Group B (252 $\mu\text{mol/L}$, range 160-480, $p= 0.0001$). Four patients in Group A experienced an early acute rejection episode (within the first three months post-transplant), but these were steroid responsive and all patients maintained excellent graft function throughout, with no graft losses. In Group B, twelve patients experienced acute rejection: four patients had a single early acute rejection episode and one of these was steroid resistant requiring OKT3 therapy; five

patients had an acute rejection episode after the first three months post-transplant; and three patients experienced multiple acute rejection episodes. Fifteen patients in Group B had biopsy-proven chronic rejection, six of whom had no history of acute rejection and there were five graft losses as a result of chronic rejection during the course of this study (Table 3.1). The immunosuppressive regimens shown in Table 3.2 illustrate that more patients received cyclosporin as baseline therapy in Group B compared with Group A (16 versus 11) and more patients were on a triple regimen in Group B compared with Group A (7 versus 4), but these differences were not statistically significant.

DONOR-SPECIFIC PROLIFERATIVE RESPONSES IN VITRO

To ensure that donor cells were sufficiently viable to act as stimulators in MLR, their capacity to induce proliferation in a normal individual was tested in parallel with cells from the recipient. Patients were excluded from the analysis if the donor or recipient cell viability was poor, cell count too low or if the donor cells were unable to induce proliferation in a normal control. As an additional check, some patient samples were repeated to check for reproducibility of results. Pooled cells from five normal volunteers were used as the third party control for all patients, as described in Chapter 2. Mixed lymphocyte reactions were performed to assess the proliferative response of each patient to their respective donor, third party cells and PHA. Parallel plates were set up for cytokine analysis by ELISA and RT-PCR.

Donor-specific hyporesponsiveness, as defined by an RRI in MLR of $\leq 20\%$, was detected in 18/36 (50%) patients overall. This consisted of 12/18 (67%) patients

in Group A and 6/18 (33%) patients in Group B (Figure 3.1a), but this difference was not statistically significant (X^2 test, $p=0.157$). The mean RRI of patients in Group A (14.7 ± 3.2) was significantly lower than that of Group B (36.7 ± 5.8 , $p=0.003$). All patients showed good proliferative responses after stimulation with third party cells (Figure 3.1b) or PHA (Figure 3.1c) and there were no significant differences in these responses between the two patient groups. DSH was detected in six patients in Group B – four with chronic rejection, one with steroid-resistant acute rejection and one with multiple episodes of acute rejection. Therefore, DSH was not confined to patients with good allograft outcome.

The immunosuppressive regimes in patients who showed DSH and those who did not were similar, as 15/18 (83%) patients with DSH and 13/18 (72%) patients without DSH had been maintained on cyclosporin-based immunosuppression (X^2 test, $p>0.05$). There was a significant correlation between the relative response index and serum creatinine ($r=0.531$, $p<0.001$) when all patients of Groups A and B were taken together (Figure 3.2). Twelve patients had both good graft function (serum creatinine $< 140 \mu\text{mol/L}$) and DSH ($\text{RRI} \leq 20\%$, dotted lines in Figure 3.2).

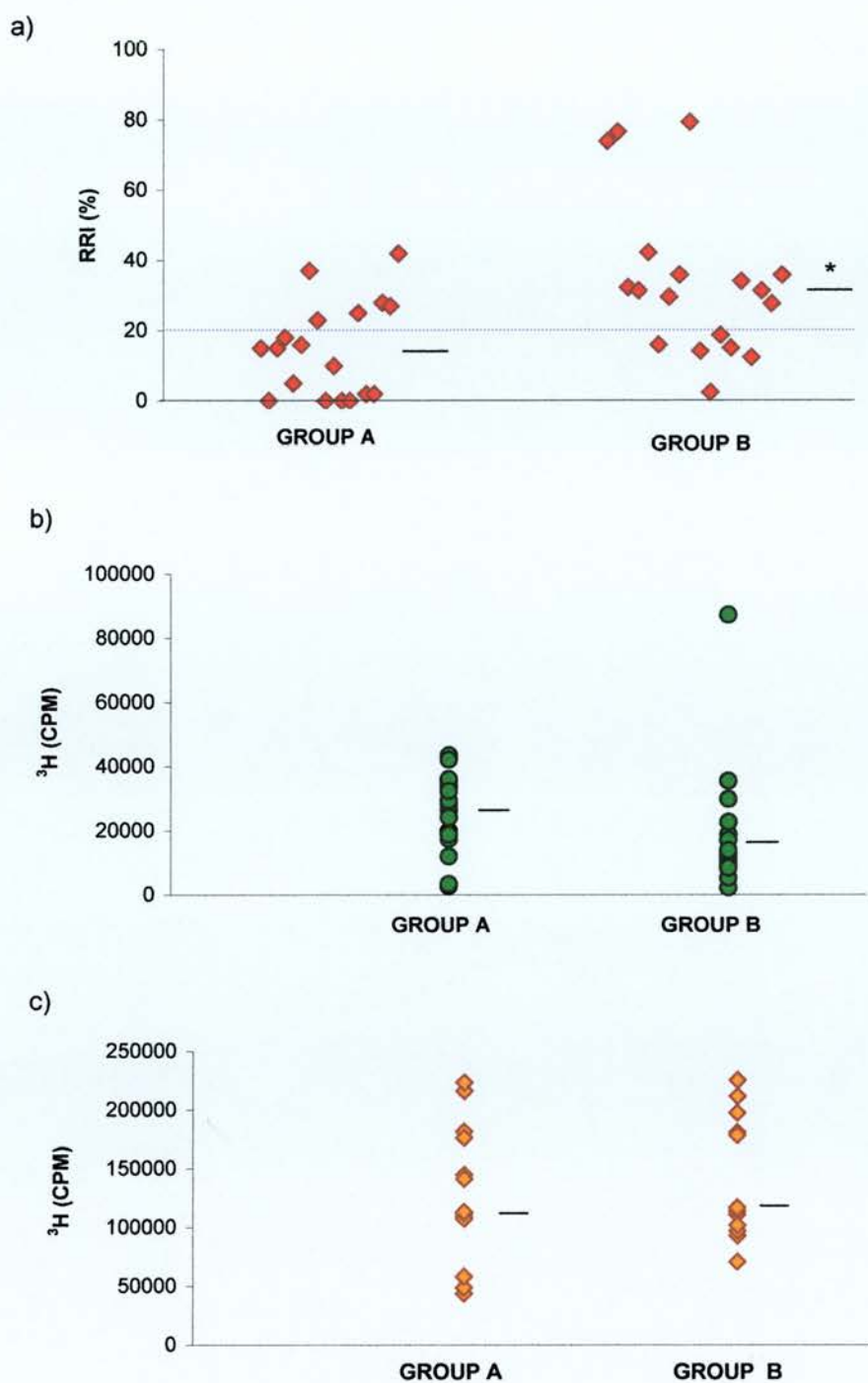


Figure 3.1: Proliferative responses of cadaveric recipients in Groups A (n=18) and B (n=18). Results shown are the relative response indices (RRI) of each group with DSH represented by the dotted line (a), (b) and (c) show the mean ^3H -TdR incorporation of triplicate cultures of recipient responder cells after stimulation with third party cells or PHA respectively. Group means are represented by the lines, *p<0.05.

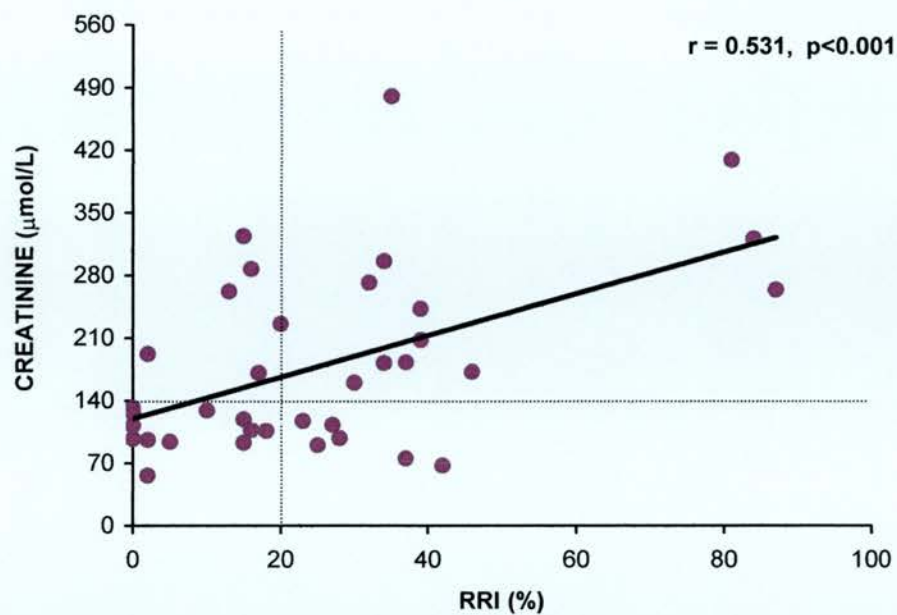


Figure 3.2: Correlation between the relative response index (RRI) and renal function as determined by serum creatinine for Group A and B patients combined (n=36). Dotted lines separate the patients with donor-specific hyporesponsiveness (RRI \leq 20%) and good graft function (creatinine < 140 μ mol/L).

PRODUCTION OF CYTOKINES

The cytokine profiles of patients in Group A were compared with patients in Group B to determine whether there was a correlation with graft outcome. Lymphocytes from patients in Group A produced significantly less IL-2 ($p=0.017$) and significantly more IL-4 ($p=0.015$) than from patients in Group B when stimulated with donor cells (Figure 3.3a). The two groups produced similar levels of IL-2 and IL-4 following third party stimulation (Figure 3.3b). Although all patients showed higher levels of IL-2 production in response to third party stimulator cells than in response to autologous stimulators, recipients in Group A produced significantly less IL-2 in response to their respective donors (Figures 3.4a and 3.4b) compared with the levels produced following third party stimulation. Some patients in Group A also produced higher levels of IL-4 in response to donor cells than in response to third party cells (Figure 3.4c). Patients in Group B showed no differences in IL-2 or IL-4 production after donor or third party stimulation (Figures 3.4b and 3.4d). There were no significant differences in IL-10 or IFN- γ production between the two groups following either donor (Figure 3.3a) or third party stimulation (Figure 3.3b).

When all patients were considered together, there was a significant correlation between the RRI and both IL-2 ($r=0.53$, $p<0.001$, Figure 3.5a) and IFN- γ production ($r=0.48$, $p<0.01$, Figures 3.5b). There was also a significant negative correlation between the RRI and IL-4 production ($r= -0.33$, $p<0.01$, Figure 3.5c), but no correlation between IL-10 production and RRI (Figure 3.5d). No data are available on TGF- β production in this cohort as the TGF- β ELISA was not established at the time when these patients were analysed and could not be performed retrospectively as the supernatants contained human AB serum.

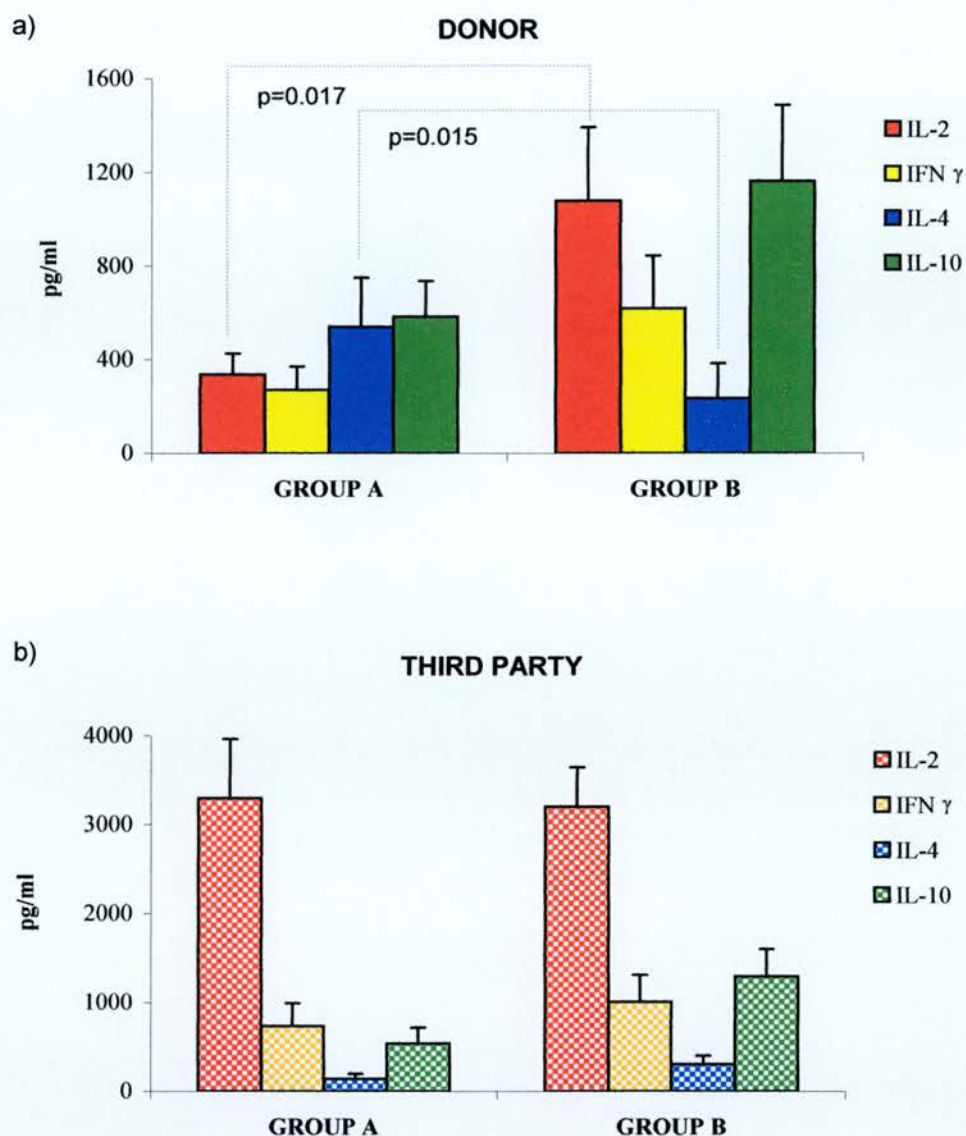


Figure 3.3: Cytokine production by patients in Group A (n=18) and Group B (n=18) in response to stimulation with donor (a) or third party (b) stimulator cells. Results shown are mean cytokine production \pm SEM after subtraction of the background responses in the presence of autologous stimulators.

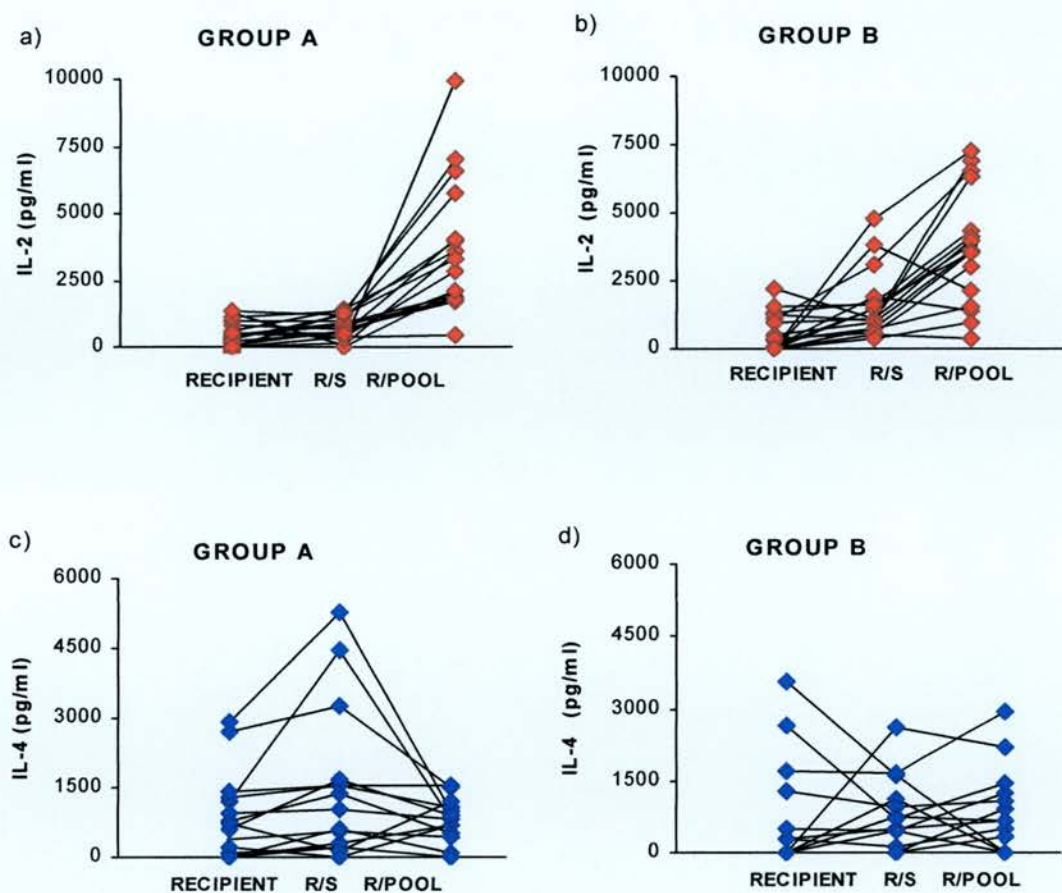


Figure 3.4: Production of IL-2 and IL-4 by Group A (n=18) and B (n=18) recipients after stimulation with autologous (recipient), donor (R/S) or third party (pool) cells. Results shown are the mean concentrations of cytokines from triplicate samples from individual patients.

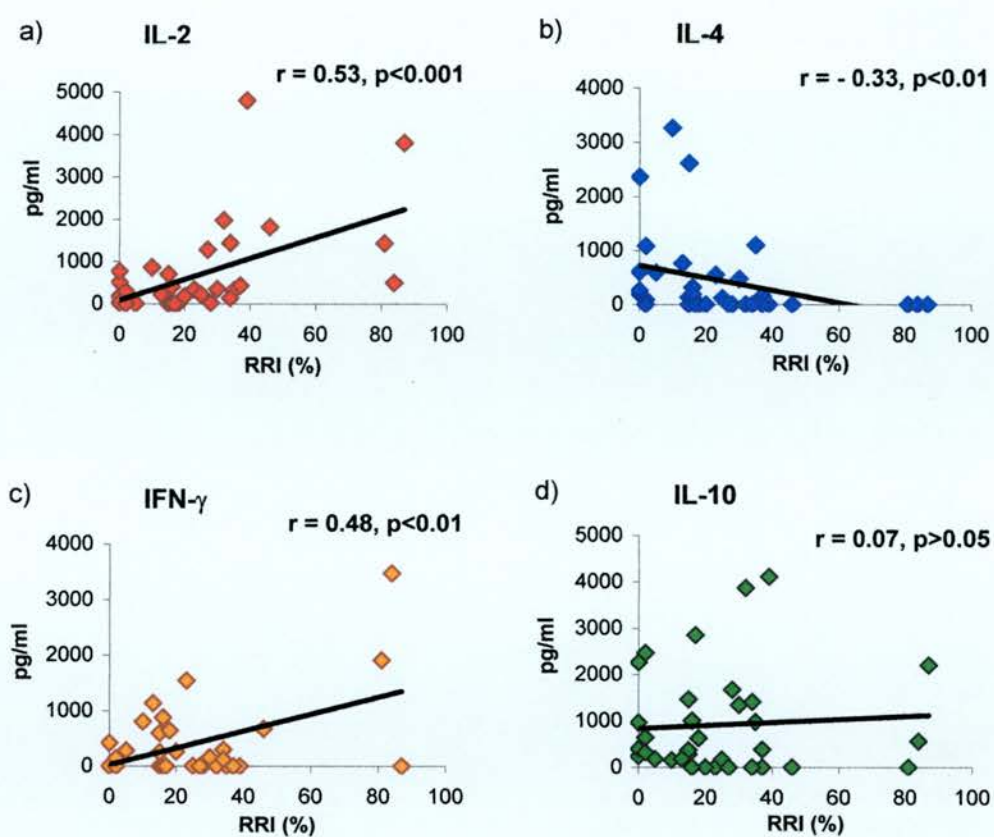


Figure 3.5: Correlation between the relative response index (RRI) and donor-specific cytokine production in patients from Groups A and B combined (n=36).

EXPRESSION OF CYTOKINE mRNA

Fourteen patients in the Cadaveric Cohort, seven from each group, were analysed by RT-PCR to give a sensitive indication of de novo cytokine production to assist interpretation of the ELISA data. Only a limited number of patients could be analysed in this way due to inadequate cell numbers and time constraints. In addition, there was insufficient cDNA for some patients to analyse all the cytokines and in these cases, I used PCR to confirm the absence of cytokine production when no protein secretion could be detected by ELISA. The data for patients in Group A is shown in Figure 3.6 and for Group B in Figure 3.7.

IL-2

IL-2 was assessed in thirteen patients. Gene expression was detected in three patients, one from Group A (CD, Figure 3.6) and two from Group B (FH and SP, Figure 3.7). Gene expression was not detected in eight patients in whom donor-specific cytokine protein was secreted, three from Group A (GS, AS, and IPt, Figure 3.6) and five from Group B (GT, MB, SH, BN and AK, Figure 3.7). All the patients who produced no measurable cytokine protein also showed no gene expression. IL-2 expression could not be assessed in two patients, both from Group A (JJ and IP, Figure 3.6), as β -actin mRNA could not be detected when these cells were stimulated with donor stimulators.

IFN- γ

IFN- γ gene expression was determined for thirteen patients, six from Group A and seven from Group B. IFN- γ was detected in six patients, one from Group A (GS,

Figure 3.6) and five from Group B (GT, FH, MB, AK, and SP Figure 3.7). IFN- γ message was detected in one patient (AS, Figure 3.6) in whom no protein was secreted, although in one other patient, no message was detected when cytokine protein was produced (IPt, Figure 3.6). IFN- γ message was not detected in three patients in whom no protein was measure by ELISA, one from Group A (JR, Figure 3.6) and two from Group B (SH and BN, Figure 3.7). IFN- γ expression could not be assessed in two patients, both from Group A (JJ and IP, Figure 3.6), as β -actin mRNA could not detected when these cells were stimulated with donor stimulators.

IL-4

IL-4 mRNA proved to be very difficult to detect, despite multiple attempts to optimise PCR conditions with different primers and no reliable data were obtained.

IL-10

IL-10 gene expression was analysed in only six patients, three from each group. Of these, IL-10 was detected in three patients when cytokine protein was measured by ELISA, one from Group A (GS, Figure 3.6) and two from Group B (FH and SP, Figure 3.7). IL-10 message was detected in one patient in whom no cytokine protein was measured by ELISA (GT, Figure 3.7). The assay was unsuccessful in one patient (AS, Figure 3.6). No gene expression was detected in one patient who produced no cytokine protein (JR, Figure 3.6).

TGF- β

TGF- β mRNA expression was assessed in thirteen patients, six from Group A and seven from Group B. Many patients in both groups appeared to show donor-specific TGF- β mRNA expression (GS, AS and IPt, Figure 3.6; all patients in Group B, Figure 3.7), but this was also found in cells stimulated with autologous cells (lane 1), third party stimulators (lane 3), and PHA (lane 4). As noted earlier, TGF- β protein secretion was not measured in this cohort of patients.

Although these results showed that cytokine gene expression frequently corresponded with the presence of cytokine protein as measured by ELISA, this was not always the case and several instances occurred in which the two techniques gave discordant results. Because of this, and because of technical problems with several of the PCR methods, I did not pursue PCR analyses in other groups.

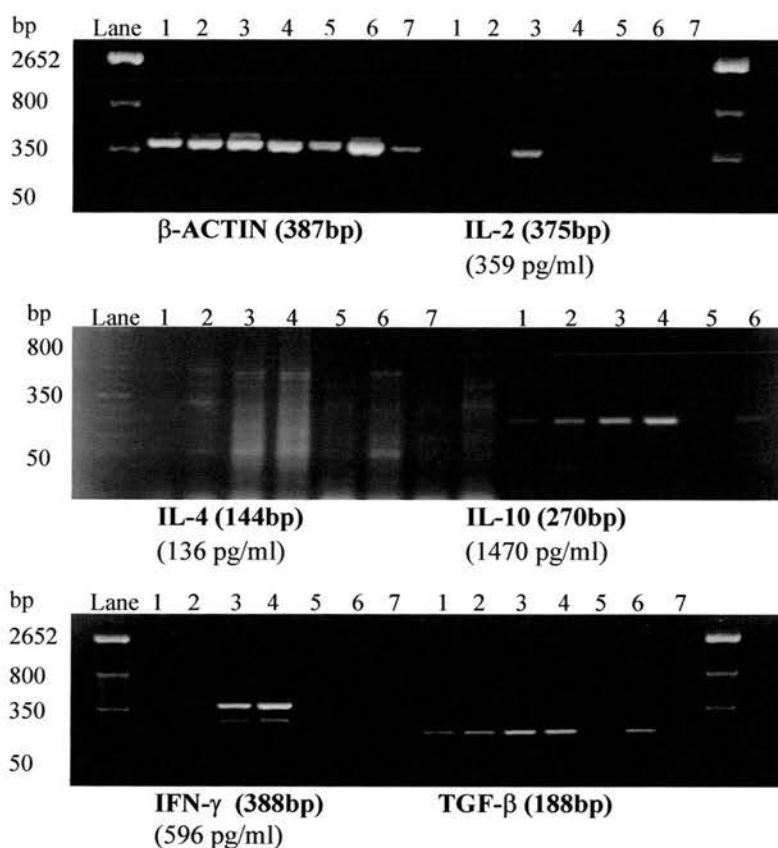


Figure 3.6a: GS

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

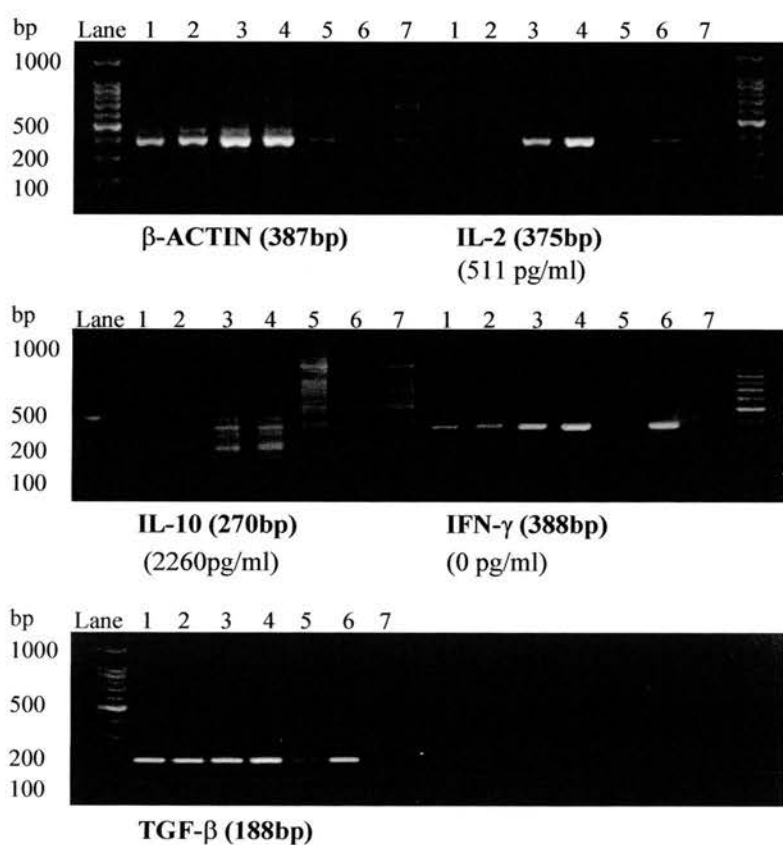


Figure 3.6b: AS

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

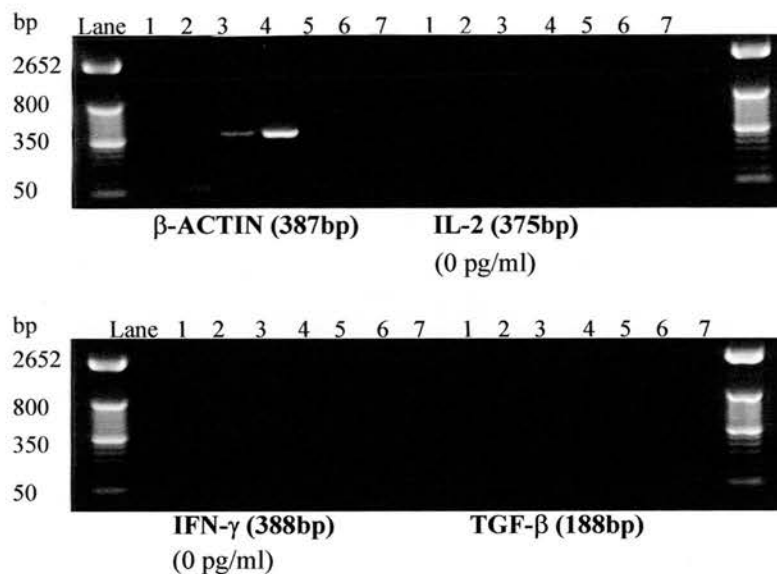


Figure 3.6c: JJ

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

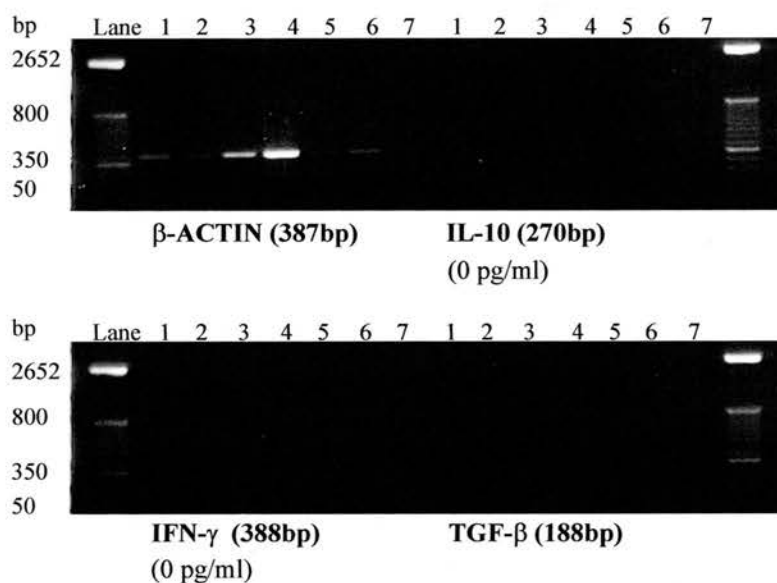


Figure 3.6d: JR

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

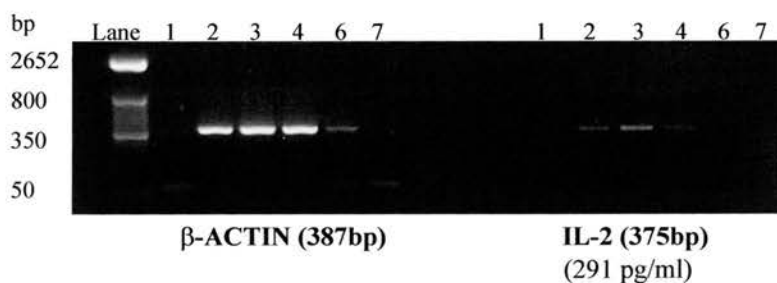


Figure 3.6e: CD

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 6: Pool
Lane 7: Neg Control

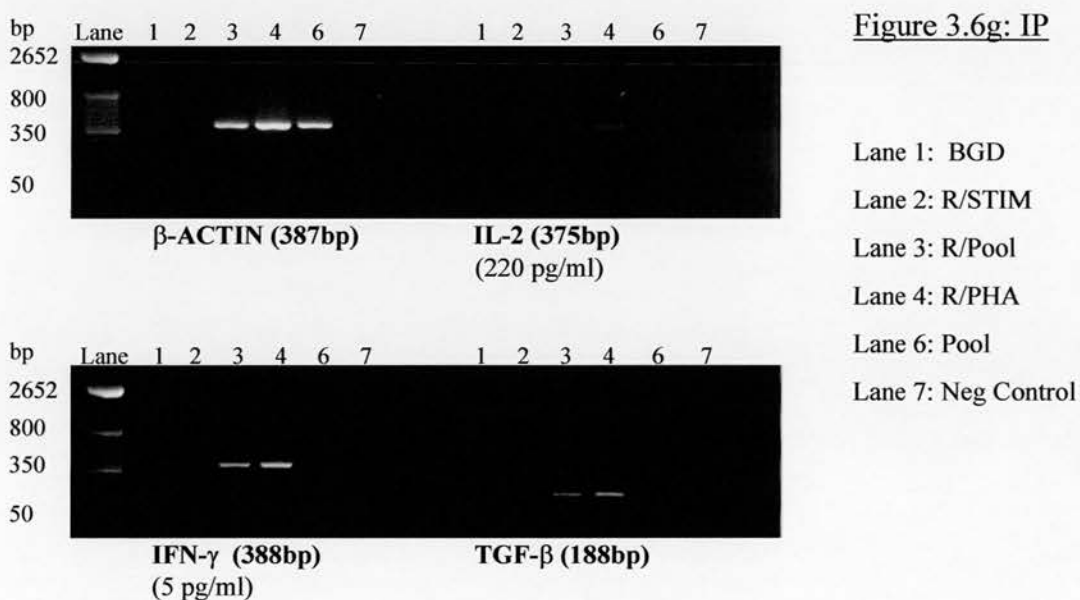
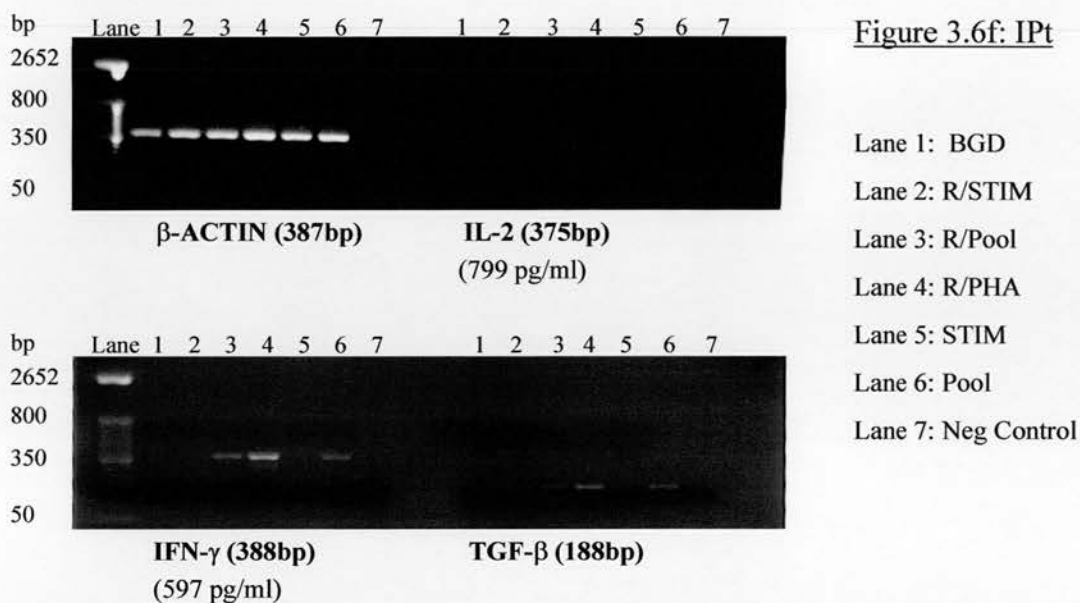


Figure 3.6 (a-g): IL-2, IL-4, IL-10, IFN-γ and TGF-β mRNA expression measured by RT-PCR in cells from patients from Group A. Lane 1 - autologous control (BGD), Lane 2 - donor-stimulated cells (R/S), Lane 3 - Third party stimulated cells (R/Pool), Lane 4 - PHA stimulated cells(R/PHA), Lane 5 - stimulator cells (STIM, insufficient for some patients), Lane 6 - Third party cells (Pool), Lane 7 - negative control. The levels of donor-specific cytokine protein measured by ELISA in each patient are shown where appropriate.

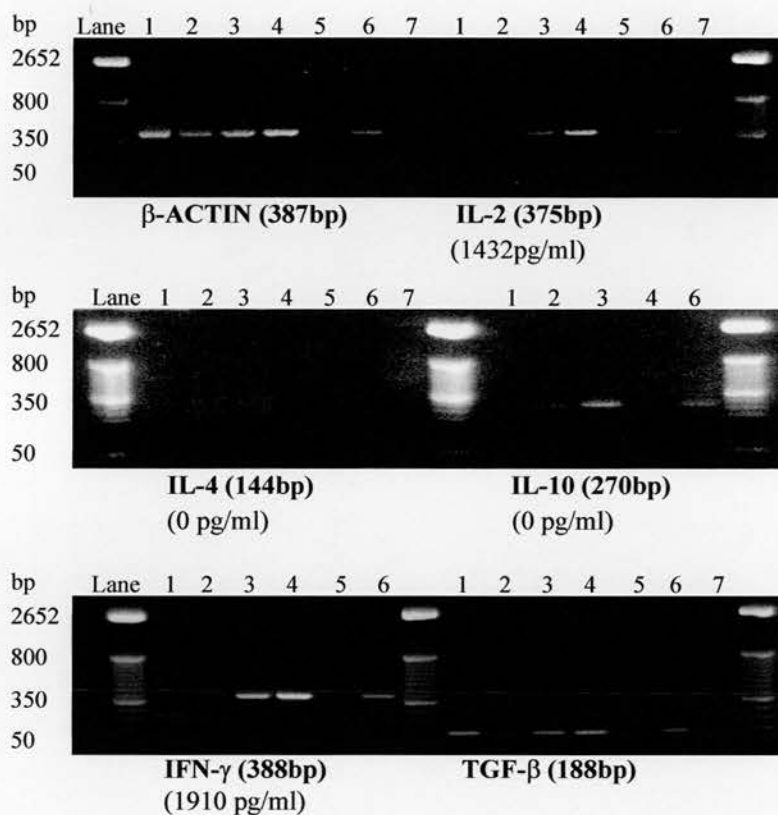


Figure 3.7a: GT

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

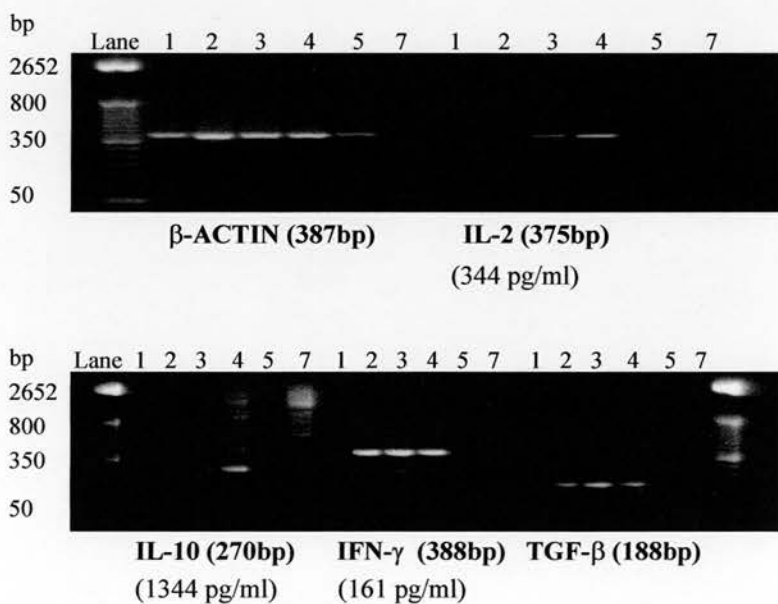


Figure 3.7b: FH

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 7: Neg Control

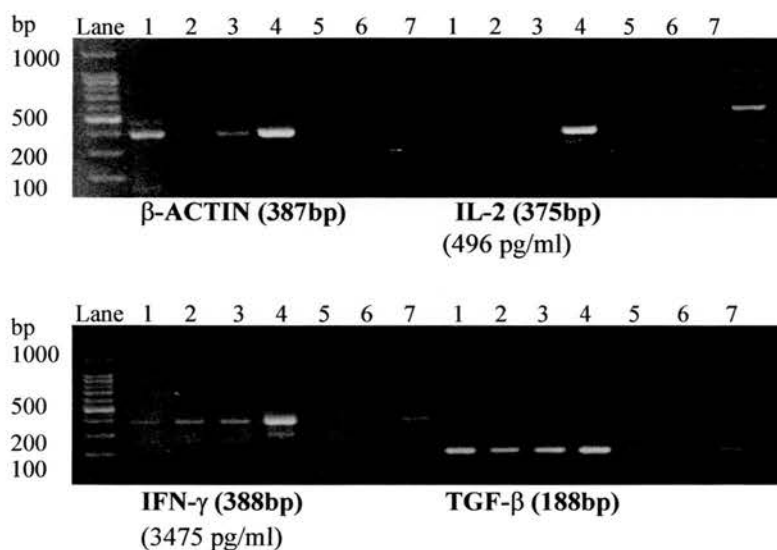


Figure 3.7c: MB

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Third Party
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Third Party
Lane 7: Neg Control

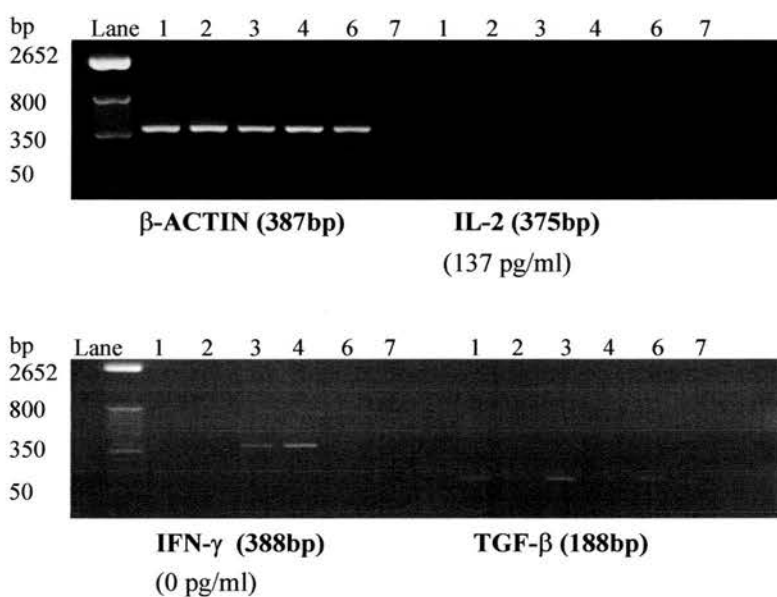


Figure 3.7d: SH

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 6: Pool
Lane 7: Neg Control

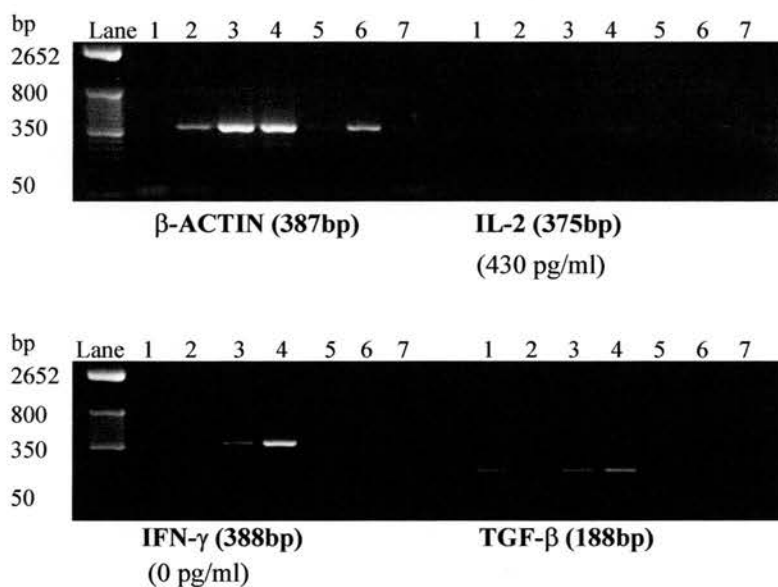


Figure 3.7e: BN

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Pool
Lane 7: Neg Control

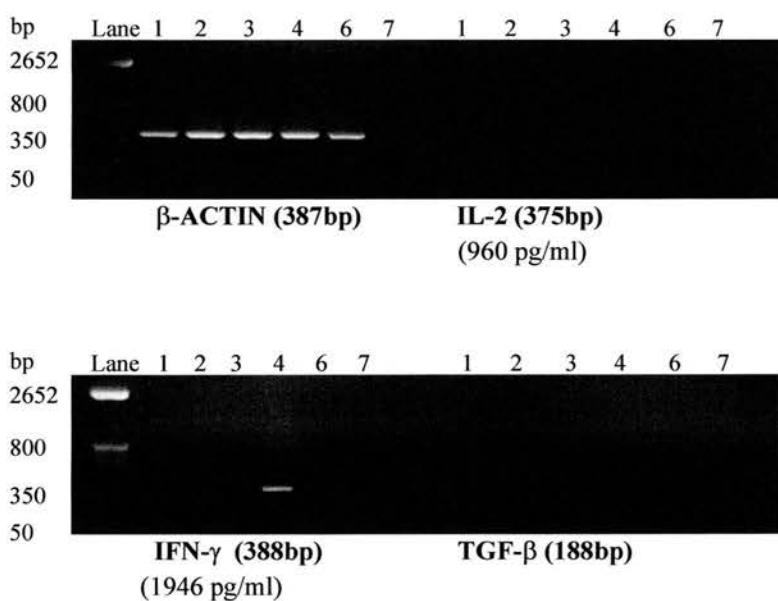


Figure 3.7f: AK

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Pool
Lane 4: R/PHA
Lane 6: Pool
Lane 7: Neg Control

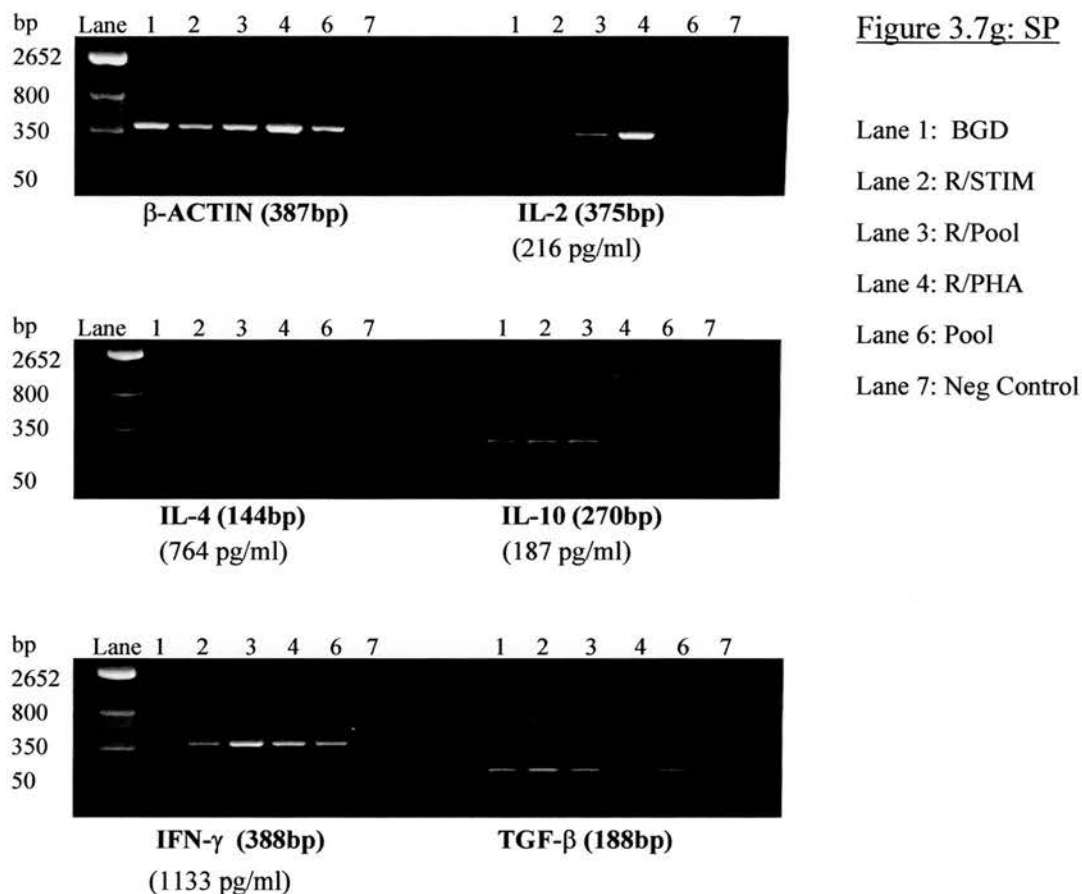


Figure 3.7 (a-g): IL-2, IL-4, IL-10, IFN- γ and TGF- β mRNA expression measured by RT-PCR in cells from patients from Group B. Lane 1 - autologous control (BGD), Lane 2 - donor-stimulated cells (R/S), Lane 3 - Third party stimulated cells (R/Pool), Lane 4 - PHA stimulated cells (R/PHA), Lane 5 - stimulator cells (STIM, insufficient for some patients), Lane 6 - Third party cells (Pool), Lane 7 - negative control. The levels of donor-specific cytokine protein measured by ELISA in each patient are shown where appropriate.

CLINICAL FEATURES OF DSH

Clinical parameters were assessed to determine whether patients who showed DSH had more favourable pre-transplant criteria (including better HLA-matching and lower levels of PRA), more intensive immunosuppression, or better post-transplant graft function than patients without DSH. When patients were divided according to the presence of or absence of DSH ($\text{RRI} \leq 20\%$), irrespective of graft function, no significant difference was found with respect to age, time since transplant, or degree of total HLA-mismatch (Table 3.3). However, patients who showed DSH appeared to be better matched at HLA-DR than those without DSH although this did not reach statistical significance (Table 3.4). Although patients with DSH appeared to have better graft function than those without DSH (median serum creatinine $115 \mu\text{mol/l}$ versus $226 \mu\text{mol/l}$), this was not statistically significant ($p=0.199$). The immunosuppressive therapy load was comparable, with 13/18 patients with DSH and 12/18 without DSH on dual therapy (prednisolone and either azathioprine or cyclosporin), and 5/18 with DSH and 6/18 without DSH on triple therapy (prednisolone, azathioprine and cyclosporin).

IMMUNOLOGICAL FEATURES OF DSH

Next, I compared the cytokine profiles of patients who showed DSH with those who did not, to assess whether MLR-defined DSH was associated with a particular pattern of cytokines and also to determine whether there was any correlation with graft outcome. The DSH group produced significantly less IL-2 ($p=0.001$, Figure 3.8a) than the non-DSH group when stimulated with donor cells. All patients in the DSH group produced higher levels of IL-2 in response to third party stimulation than after

stimulation with donor cells, but three patients in the non-DSH group produced higher levels of IL-2 in response to donor stimulation than after third party stimulation. Patients who showed DSH did not appear to have generalised immune suppression, as proliferation (Figure 3.8b and 3.8c) and IL-2 production (Figure 3.9a and 3.9b) in response to either third party or mitogen stimulation was similar in the DSH and non-DSH groups.

Patients who showed DSH produced significantly more IL-4 ($p=0.007$) in response to donor stimulation than those who did not show DSH. In addition, there was no significant difference in the levels of IL-4 produced following third party (Figure 3.9c) and mitogen stimulation (Figure 3.9d) in patients who showed DSH and those who did not. There were no significant differences in donor-specific IL-10 and IFN- γ production between the two groups (Figure 3.8).

To try and identify a sub-group of patients who might be candidates for reduction of immunosuppression, I analysed further those patients who showed both good allograft function and MLR-defined DSH. Twelve patients met these criteria and eight of these produced higher levels of IL-4 than IL-2 in response to donor stimulation (patients 5-12, Figure 3.10a). The remaining six patients who showed DSH had poor graft function and only two of these produced higher levels of IL-4 than IL-2 (patients 14 and 18, Figure 3.10a). In the non-DSH group, only two patients produced higher levels of IL-4 than IL-2 (patients 11 and 14, Figure 3.10b). Four patients in the DSH group and one patient in the non-DSH group produced no detectable IL-2 or IL-4 in response to donor stimulation. Therefore, a profile of low IL-2 production /high IL-4 production is seen more often in patients who show DSH, but this occurs irrespective of whether graft function is good or not.

Table 3.3: Clinical features of cadaveric recipients who showed DSH (defined as RRI \leq 20%) and those who did not.

GROUP	AGE TX (yrs)	TIME TX (yrs)	M/M	CREAT (μmol/L)
DSH (n=18)	39.4 (11.3 - 55.2)*	11 (8.5 - 17.9)	2.5 (0 - 5)	122.5 (56 - 324)
NON-DSH (n=18)	34.7 (19.3 - 62)	10 (7.9 - 15.5)	3 (1 - 5)	182.5 (67 - 480)
p value	0.711	0.163	0.116	0.199

AGE TX: age at transplant, TIME TX: time interval since transplant.

M/M: degree of HLA-mismatch, CREAT: serum creatinine.

*Results are shown in medians and ranges. (Wilcoxin signed rank test).

Table 3.4: Degree of HLA-mismatch in cadaveric recipients with and without DSH.

HLA-DR MISMATCH	DSH (n=18)	NON-DSH (n=18)	p (Fisher's Exact Test)
0	12	6	0.066
1	5	10	0.176
2	1	2	0.229

DSH: donor-specific hyporesponsiveness

NON-DSH: donor-specific hyporesponsiveness not shown

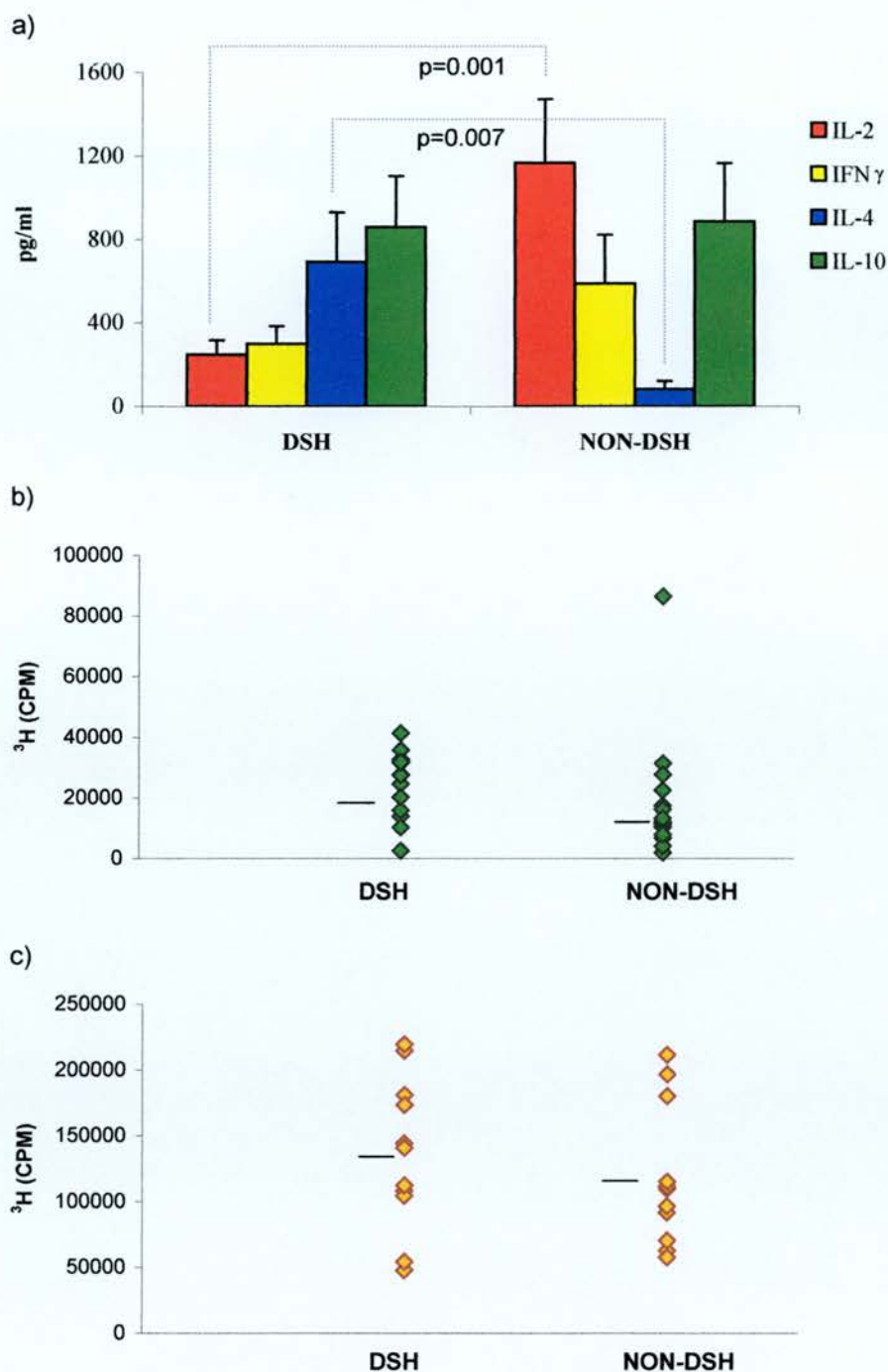


Figure 3.8: Immunological parameters of donor-specific hypo-responsiveness. Donor-specific cytokine production (a) and proliferative response to third party stimulator cells (b) and PHA (c) in patients who showed DSH (defined as $\text{RRI} \leq 20\%$) in MLR ($n=18$) compared with those who did not ($n=18$). Results shown are mean concentrations of cytokines \pm SEM for the two groups after subtraction of the background responses in the presence of autologous stimulators (a), and the mean ^3H -TdR incorporation for triplicate cultures of individual recipient responder cells to stimulation with third party cells (b) and PHA (c). Group means are shown by the lines.

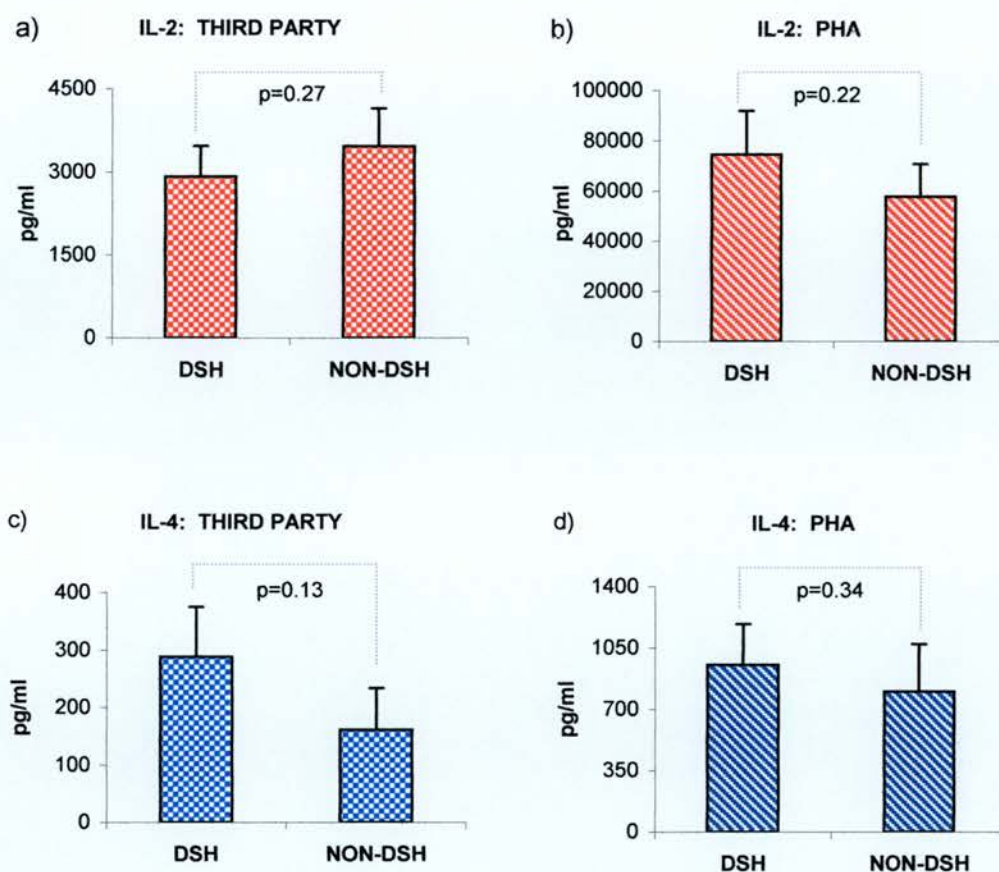


Figure 3.9: IL-2 and IL-4 production by recipients in response to stimulation with third party cells (a and c) and PHA (b and d) in patients who showed DSH (defined as $RRI \leq 20\%$) (n=18) and those who did not (n=18). Results shown are means \pm SEM for each group.

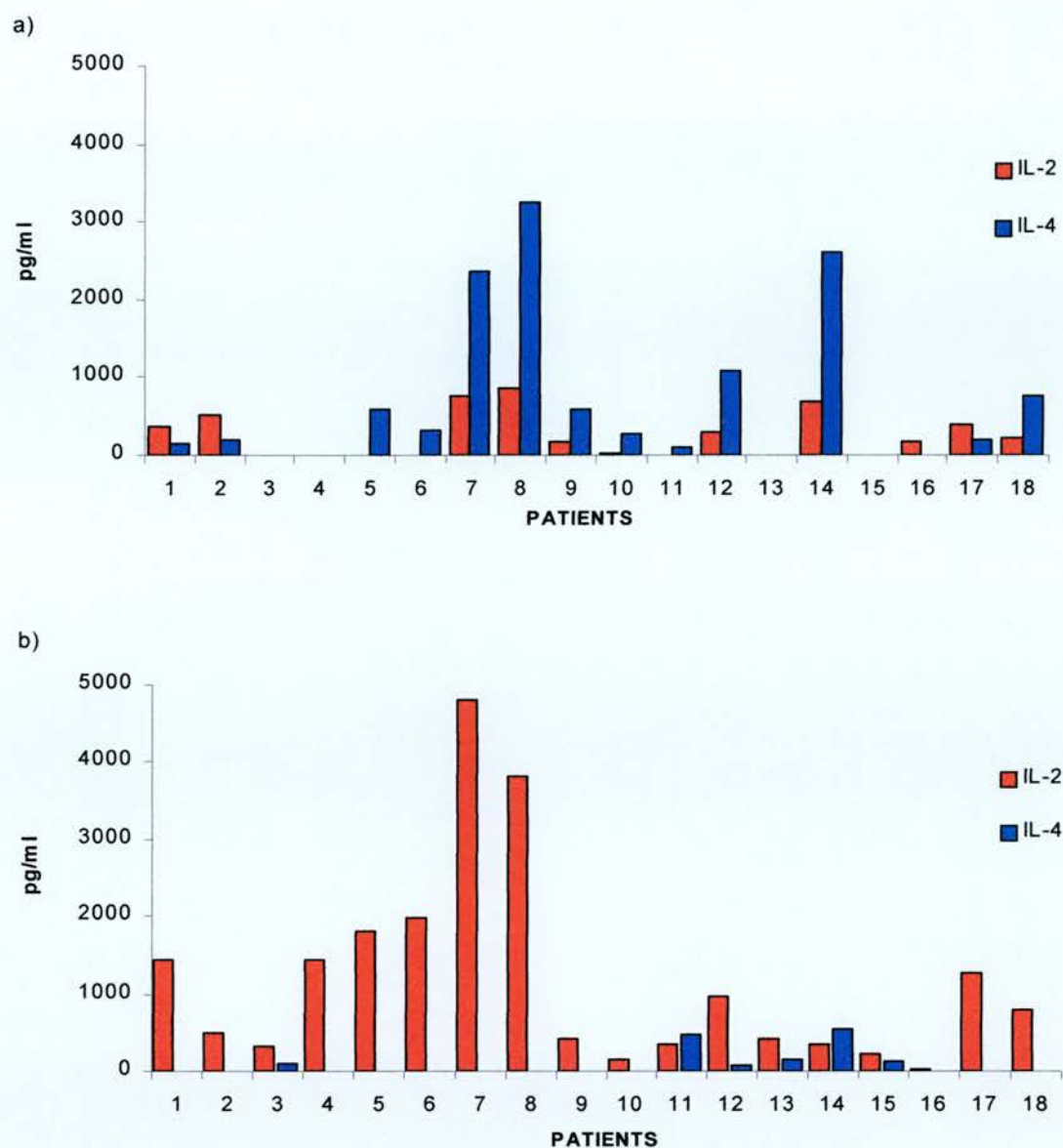


Figure 3.10: Donor-specific IL-2 and IL-4 production in patients who showed DSH (n=18) (a) and those who did not show DSH (n=18) (b), as defined by a RRI \leq 20%. Results shown are mean cytokine concentrations of triplicate cultures from individual patients.

RE-DEFINITION OF DSH

I initially analysed my data by defining the presence of DSH as an RRI of $\leq 20\%$ between donor and third party stimulation. This was in line with previous publications (Colombe et al, 1989; Ghobrial, 1994; Kim, 1996; Salomao et al, 1998; Ishido, 1999) and the practice in the local Tissue Typing laboratory. However, multiple definitions have been applied to define DSH in previous studies and most of these have been performed prospectively with short-term follow-up. As my work involved a single time point analysis of long-term graft survivors in a retrospective study, it was possible that a different threshold for defining DSH may be required. To test whether an RRI of $\leq 20\%$ was an appropriate definition of DSH for my data set, I constructed a receiver operating characteristic (ROC) curve, using the method described by Altman et al, 1994, to determine the optimal cut-off that maximised both sensitivity and specificity of the index (Figure 3.11). The specificity of the test was unchanged at 67% between a cut-off of 20-28%, but the sensitivity rose from 67% at a cut-off of 20% to 89% at a cut-off of 28%. I also calculated the positive and negative predictive values of the assay at each possible cut-off value of RRI (Table 3.5). A cut-off value of 28% yielded higher positive and negative predictive values for the assay compared with my previous definition using a cut-off of 20%.

When I used an RRI of $\leq 28\%$ as a redefinition of DSH, 16/18 (89%) patients with good graft outcome (Group A) and 6/18 (33%) with poor graft outcome (Group B) showed DSH ($p=0.005$, Figure 3.12a). The level of graft function in the two groups was also now significantly different, with a median serum creatinine of 115 $\mu\text{mol/l}$ in the DSH group compared with 226 $\mu\text{mol/l}$ in the non-DSH group ($p=0.004$, Table 3.6). There was still no significant difference in the median age (39.4 versus

34.6 years, $p=0.3$) or duration since transplant (10.8 versus 10 years, $p=0.18$) in the DSH and NON-DSH groups respectively. Although there was no significant difference in the total HLA-mismatch between the two groups, DSH was again associated with better HLA-DR matching, as 13/18 patients who had no mismatches at the HLA-DR locus showed DSH ($p=0.017$, Fischer's Exact Test). Equal numbers of patients were maintained on cyclosporin based immunosuppression (73% versus 79%) in the DSH and NON-DSH groups respectively. There was no significant difference in the incidence of acute rejection episodes between patients who showed DSH compared with those who did not (50% versus 43%), however significantly fewer patients who showed DSH experienced chronic rejection (18% versus 79%, $p=0.003$) (Table 3.3).

The cytokine profile of patients who showed DSH by this revised definition showed even lower IL-2 production ($p=0.011$) and higher IL-4 production ($p=0.01$) compared with non-DSH patients than suggested by the previous analysis (Figure 3.12b). As before, patients with DSH produced equal amounts of IL-2 in response to third party stimulation (Figure 3.13a) and higher levels of IL-2 in response to mitogen (Figure 3.13b) than patients without DSH. Although patients with DSH also produced higher levels of IL-4 in response to third-party (Figure 3.13c) and mitogenic (Figure 3.13d) stimulation, these differences were not statistically significant.

In the previous analysis, DSH defined as a $RRI \leq 20\%$, was identified in twelve patients with good graft function, and eight of these patients produced higher amounts of donor-specific IL-4 and low levels of IL-2 (Figure 3.10a). However as described above, this cytokine profile was also detected in four other patients: two

showed DSH, but had poor graft function and two did not show DSH, but one had good graft function. When DSH was defined as $RRI \leq 28\%$, sixteen patients with good graft function showed DSH (Figure 3.14a). Of these, nine patients produced higher levels of IL-4 than IL-2. Hence, one additional patient with good allograft function was identified as showing MLR-defined DSH by the revised definition. This patient (Patient 11, Figure 3.14a), had an RRI of 23% and produced almost two-fold higher levels of IL-4 than IL-2. A low IL-2/high IL-4 profile was also detected in three other patients, all of whom had poor graft function: two showed DSH (Patients 18 and 22, Figure 14a) and one patient did not show DSH (Patient 11, Figure 3.14b).

In order to assess the relative production of donor-specific IL-4 and IL-2 in patients who showed DSH and those who did not, the ratio of IL-4/ IL-2 was determined in each group. When all patients were considered, the ratio of IL-4/ IL-2 production in patients who showed DSH was 2.07 compared with a ratio of 0.04 in patients who did not show DSH. The ratio of IL-4/ IL-2 in patients with DSH and those without DSH did not change significantly when the level of graft function in each group was considered (Table 3.7). Therefore, patients who showed DSH produced approximately two-fold higher levels of IL-4 than IL-2 in response to donor stimulation, even in the presence of poor graft function, and patients who did not show DSH produced a low donor-specific IL-4/ IL-2 ratio, even in the presence of good graft function. Further analysis of the sixteen patients who showed DSH and good graft function showed that only nine patients produced higher levels of donor-specific IL-4 than IL-2 and the ratio of IL-4/ IL-2 production was 3.7.

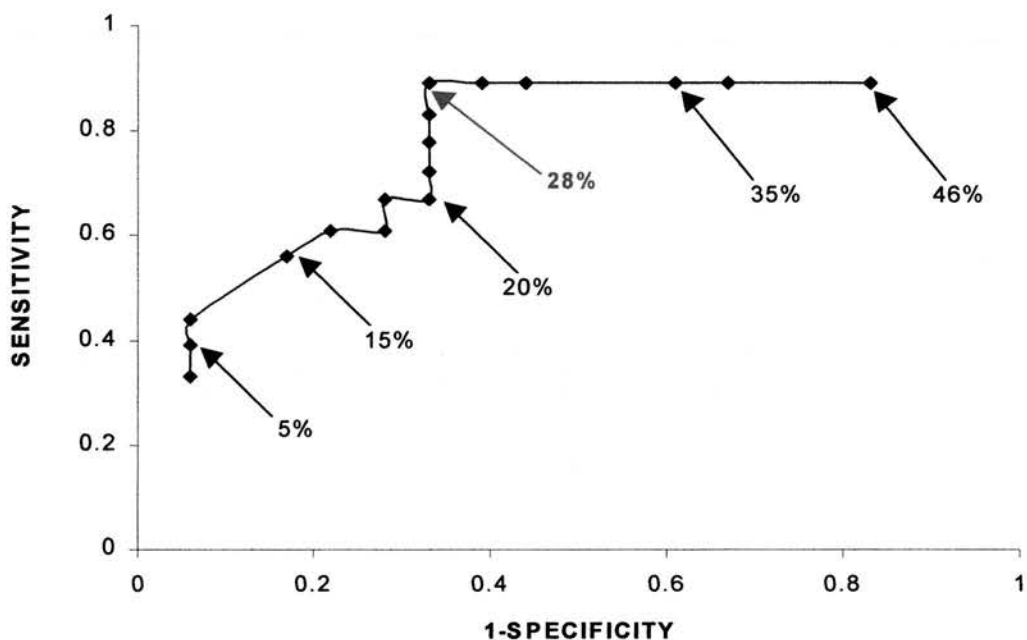


Figure 3.11: Receiver operating characteristic (ROC) curve for determining the optimal level of RRI for defining donor-specific hyporesponsiveness (DSH). Results shown are the sensitivity plotted against 1-specificity of multiple cut-off points. DSH defined by a cut-off of 28% is shown in red.

Table 3.5: The sensitivity, specificity, and predictive values of the MLR at multiple potential cut-off values of RRI.

RRI (%)	SENSITIVITY (%)	SPECIFICITY (%)	POSITIVE PREDICTIVE VALUE (%)	NEGATIVE PREDICTIVE VALUE (%)
5	39	94	88	61
10	44	94	89	63
15	56	83	77	65
18	67	72	71	68
20	67	67	67	67
25	78	67	70	75
28	89	67	73	86
30	89	61	70	85
32	89	56	67	83
35	89	39	59	78
37	89	33	55	86
42	89	17	50	75
46	89	17	48	100

RRI: Relative response index.

Table 3.6: Clinical features of cadaveric recipients who show DSH (defined as a RRI \leq 28%) and those who did not.

GROUP	AGE TX (years)	TIME TX (years)	M/M	CREAT (μ mol/L)	AR	CR
DSH (n=22)	39.4 (11.3 - 57.8)*	10.8 (8.5 - 17.9)	3 (0 - 5)	115 (56 - 324)	11	4
NON-DSH (n=14)	34.6 (19.3 - 62)	10 (7.9 - 15.5)	3 (1 - 5)	226 (67 - 480)	6	11
p value	0.3	0.18	0.1	0.004	0.67	0.0003

AGE TX: age at transplant, TIME TX: time interval since transplant, M/M: degree of HLA-A, B and DR-mismatch, CREAT: creatinine.

AR: acute rejection, CR: chronic rejection.

DSH: donor-specific hyporesponsiveness.

NON-DSH: donor-specific hyporesponsiveness not shown.

*Results are shown in medians and ranges.

Table 3.7: Ratio of donor-specific IL-4/ IL-2 production in cadaveric recipients who showed DSH and those who did not.

DSH			NON-DSH		
ALL n=22	GOOD F’N n=16	POOR F’N n=6	ALL n=14	GOOD F’N n=2	POOR F’N n=12
2.07	1.98	2.39	0.04	0.13	0.04

DSH: donor-specific hyporesponsiveness.

NON-DSH: donor-specific hyporesponsiveness not detected.

ALL: all patients in each cohort.

GOOD F’N: good graft function.

POOR F’N: poor graft function.

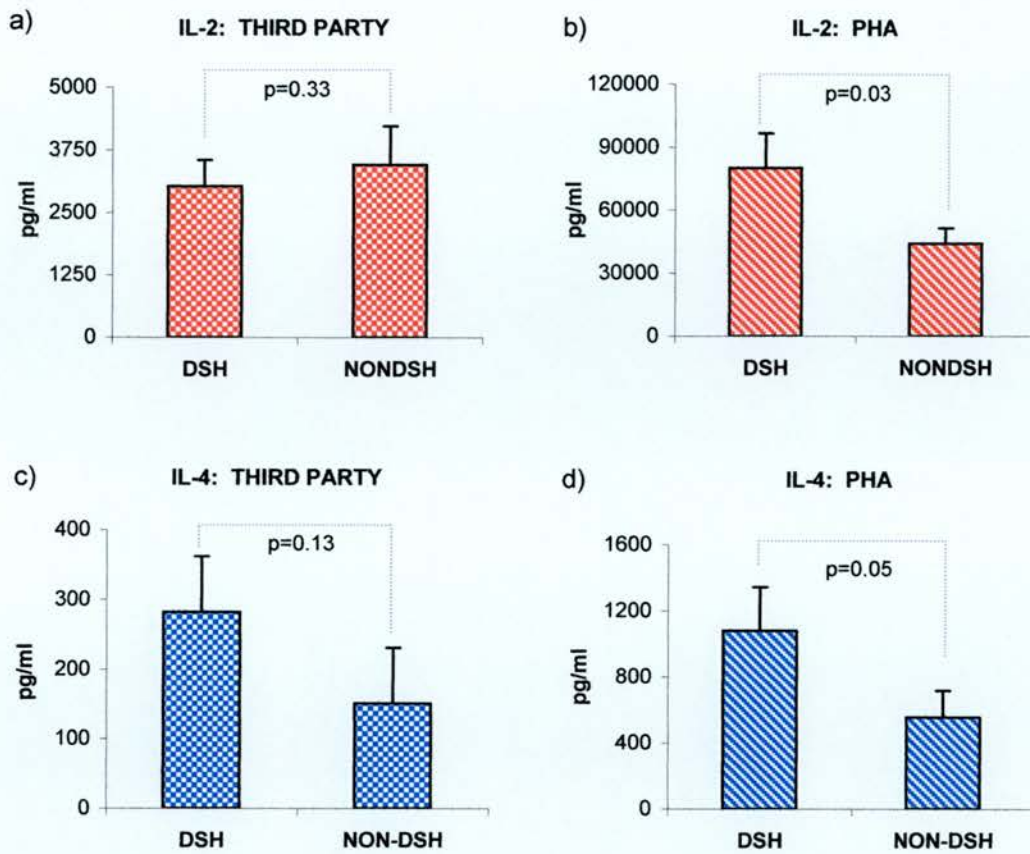


Figure 3.13: IL-2 and IL-4 production by recipients in response to stimulation with third party cells (a and c) and PHA (b and d) in patients who showed DSH (defined as RRI \leq 28%) (n=22) and those who did not (n=14). Results shown are means \pm SEM for each group.

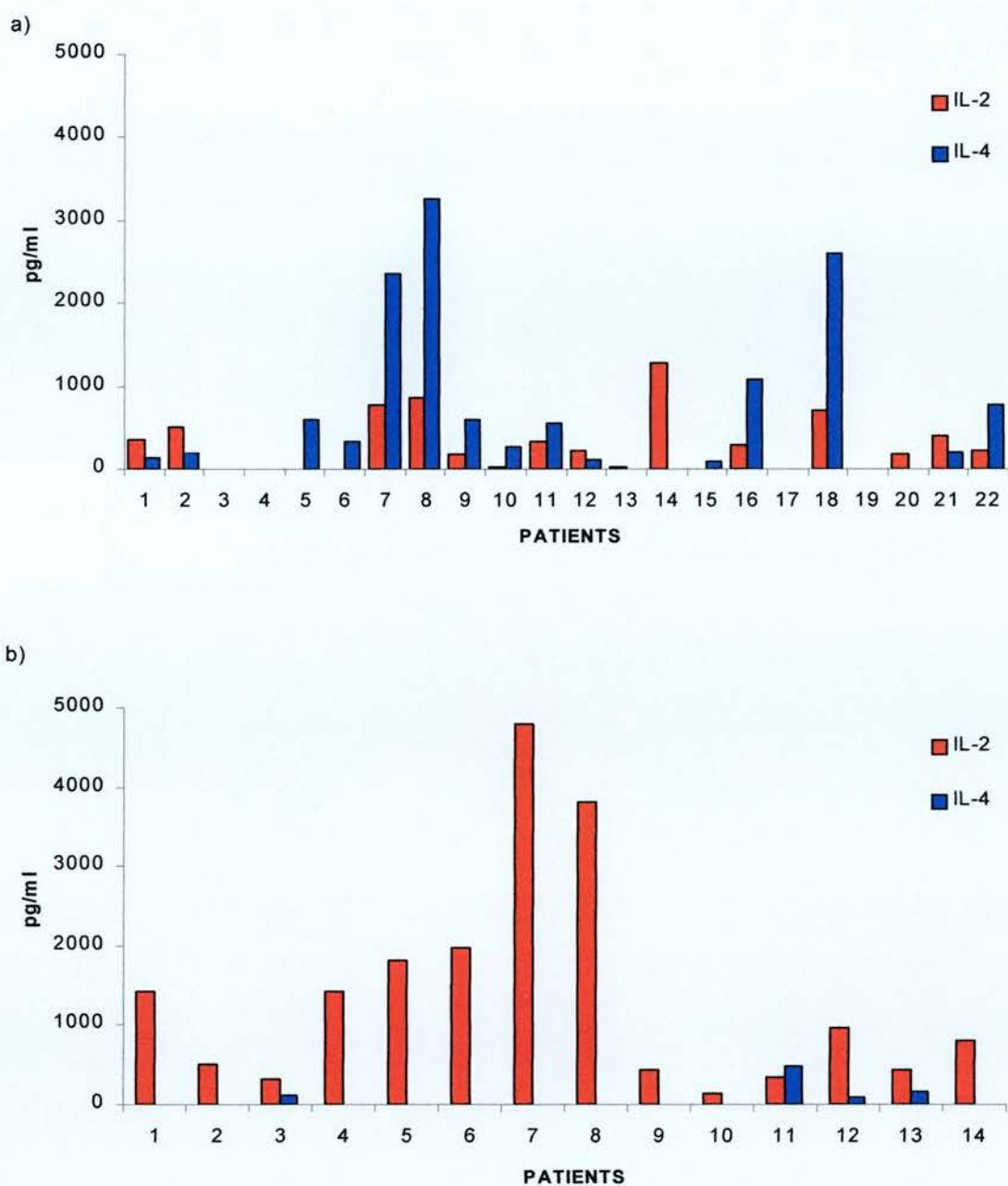


Figure 3.14: Donor-specific IL-2 and IL-4 production in patients who showed DSH (n=22) (a) and those who did not show DSH (n=14) (b), as defined by a RRI \leq 28%. Results shown are mean cytokine concentrations of triplicate cultures from individual patients.

SUMMARY

This was a retrospective study of thirty-six cadaveric allograft recipients with long-term graft survival and varied graft outcome. Patients in Group A had an uncomplicated course with good allograft outcome and patients in Group B had a poor allograft outcome with five patients returned to dialysis following graft failure. In the latter group, late acute rejection and multiple or steroid-resistant episodes had an impact on long-term graft function and may have also contributed to the development of chronic allograft nephropathy.

This cohort of patients provided the main foundation of the retrospective study and showed that donor-specific hyporesponsiveness and good graft outcome correlated with the production of low levels of donor-specific IL-2 and high levels of IL-4. A sub-group of patients with stable graft function was identified that showed DSH with this favourable cytokine profile. To maximise the sensitivity and specificity of the test, DSH was redefined as a RRI $\leq 28\%$ and this value was used for the analysis of the living-related (Chapter 4) and malignancy (Chapter 5) cohorts of the retrospective study and for the prospective cohort (Chapter 6).

CHAPTER 4: LIVING-RELATED COHORT

INTRODUCTION

Reduction of immunosuppression based on in-vitro assays has only been attempted in living-related recipients, who have longer allograft survival due to better HLA matching and shorter cold ischaemia after organ retrieval and hence are exposed to immunosuppression for longer (Fletcher et al, 1989; Kerman et al, 1997). Despite the fact that MLR-defined DSH is a common finding in such recipients, it has proved difficult to use it as a reliable predictor of adverse events and acute rejection may still occur following reduction of immunosuppression (Fletcher et al, 1989). Having shown in Chapter 3 that a value of an RRI $\leq 28\%$ may be a better definition of DSH and that donor-specific cytokine production may improve the discrimination between good and poor graft outcome in cadaveric recipients, I went on to apply these parameters in living related allograft recipients. This chapter describes experiments in which I examined the correlation between graft outcome, MLR-defined DSH and cytokine production in these patients.

PATIENTS

Fourteen patients (4 male and 10 female) were included. The numbers in this group are relatively small due to difficulty in obtaining blood samples from the living donors, many of whom were lost to follow-up, had moved out of the area, or had died. All patients were first graft recipients with a functioning graft for more than four years. The aetiology of renal failure was: chronic pyelonephritis (n=6), chronic

glomerulonephritis (n=4), adult polycystic kidney disease (n=1), Goodpasture's disease (n=1), congenital renal hypoplasia (n=1), and unknown (n=1). With respect to HLA-typing, there were eight HLA haplo-identical (HLA-HI), five HLA-identical (HLA-ID) and one non-identical (HLA-NI) recipients. One of the HLA-ID recipients received a graft from an identical twin.

Classification of Patients

As with the Cadaveric Cohort, this group of patients had a mixed allograft outcome and to allow direct comparison, I divided them into two groups using the same criteria. Patients in Group A (n=8) had a good allograft outcome, with no acute rejection beyond the first three months post-transplant and a serum creatinine ≤ 140 $\mu\text{mol/l}$ at the time of entry to the study. Patients in Group B (n=6) had a poor allograft outcome, with either late or multiple acute rejection, or chronic rejection. All rejection episodes were biopsy proven. To assess the role of HLA-matching in graft outcome, the degree of HLA-match of Groups A and B was compared (Table 4.1). The clinical features of Groups A and B are shown in Table 4.2. Good graft outcome was seen in 4/5 HLA-ID, in 3/8 HLA-HI and in the HLA-NI recipient.

Rejection History

Eight patients experienced acute rejection: five occurred early (within the first three months post-transplant), one occurred late (after the first three months post-transplant) and two patients had multiple acute rejection episodes. There were no rejection episodes among the HLA-identical recipients. Three patients, two of whom had experienced acute rejection, developed biopsy-proven chronic rejection. One

recipient of an HLA-identical graft developed chronic rejection. Two of the patients with chronic rejection subsequently lost their grafts, one of whom received a further living-related graft from another sibling.

Immunosuppression

Ten patients received conventional triple therapy consisting of prednisolone, azathioprine, and cyclosporin as baseline immunosuppression. The other four patients received dual therapy: prednisolone and azathioprine in two patients, and prednisolone and cyclosporin in two patients. Immunosuppression was modified in six patients during the post-transplant course. The identical twin recipient, who received dual therapy as a precaution, discontinued all immunosuppression three months post-transplant. Four patients were converted to tacrolimus and one patient to mycophenolate after rejection episodes.

Table 4.1: The degree of HLA-match for living-related recipients in Groups A and B.

HLA-MATCH	GROUP A	GROUP B
HLA-ID	4	1
HLA-HI	3	5
HLA-NI	1	0

ID: identical, HI: haplo-identical, NI: non-identical.

Table 4.2: Clinical features of living-related recipients in Groups A and B.

GROUP	AGE TX (years)	TIME TX (years)	CREAT ($\mu\text{mol/L}$)	ACUTE REJECTION			CHRONIC REJECTION	GRAFT LOSS
				EARLY	LATE	MULTIPLE		
A (n=8)	22.1 (16.7 – 47.2)	6.8 (4.6 – 21.4)	104 (86 – 130)	3	0	0	0	0
B (n=6)	25.6 (18.7 – 33.5)	5.4 (4.1 – 12.6)	232 (116 – 354)	2	1	2	3	2

AGE TX: age at transplantation, TIME TX: duration since transplant, CREAT: creatinine.

*Results shown are medians and ranges where appropriate.

CLINICAL FEATURES OF LIVING-RELATED COHORT

The median serum creatinine of patients in Group A was significantly lower than in Group B (104 $\mu\text{mol/l}$ versus 232 $\mu\text{mol/l}$, $p=0.028$). There were no significant differences in age ($p=0.917$) or duration since transplantation ($p=0.917$) between the two groups. Six out of eight Group A patients and all the patients in Group B, received cyclosporin as baseline immunosuppression. The two patients who did not receive cyclosporin were transplanted before cyclosporin was introduced into clinical practice.

A few comparisons can be drawn between the living-related (LRD) and cadaveric (CAD) groups. In comparison with the CAD Cohort, the LRD recipients were younger (23.5 years versus 38.4 years) and had been transplanted for a shorter duration (5.7 years versus 9.8 years), and these factors may influence the incidence of DSH. The median serum creatinine was similar in Groups A (104 $\mu\text{mol/l}$ versus 102 $\mu\text{mol/l}$) and Groups B (232 $\mu\text{mol/l}$ versus 252 $\mu\text{mol/l}$) of the LRD and CAD cohorts. There was also no significant difference in the acute rejection rate of Groups A (38% versus 22%) and Groups B (83% versus 66%) in the two cohorts.

DONOR-SPECIFIC PROLIFERATIVE RESPONSES IN-VITRO

The MLR studies were performed and analysed as described in Chapter 3, with an RRI of $\leq 28\%$ used as the definition of DSH. Using this definition, DSH was detected in 9/14 (64%) living-related recipients. This consisted of 6/8 (75%) patients in Group A and 3/6 (50%) patients in Group B ($p=0.22$, X^2 test, Figure 4.1a). The

mean RRI in Group A was $20.4 \pm 9.3\%$ and in Group B was $36 \pm 12\%$ ($p=0.33$). All patients showed good proliferative responses after stimulation with third party cells (Figure 4.1b) and PHA (Figure 4.1c). In comparison with the cadaveric cohort, there was no significant difference in the mean RRI for patients in Groups A (20.4% versus 14.7%) and Groups B (36% versus 36.7%). The living-related recipients also showed levels of proliferation in response to third party cells and PHA similar to those found previously in recipients of cadaveric grafts.

PRODUCTION OF CYTOKINES

IL-2, IL-4, IL-10, IFN- γ

Lymphocytes from living-related recipients in Group A produced significantly lower levels of IL-2 ($p=0.03$) and than those from recipients in Group B after stimulation with donor cells (Figure 4.2). Group A patients also produced lower levels of IL-4 and IL-10 than Group B, but this was not statistically significant and both groups produced similar levels of IFN- γ (Figure 4.2). Compared with Group B, patients in Group A produced higher levels of IL-2 and IFN- γ in response to stimulation with third party cells (Figure 4.3a and 4.3b) and PHA (Figure 4.3c and 4.3d), but almost equivalent amounts of IL-4 and IL-10.

Donor-specific cytokine production appeared to depend on the degree of HLA-matching. HLA-ID recipients produced virtually no cytokines in response to donor cells, except for one patient (Patient 2) who produced predominantly IL-2. This patient developed chronic rejection and subsequently lost the graft (Figure 4.4a). The identical twin recipient (Patient 4) produced only a low level of IL-4

following stimulation with donor cells and no other cytokines. The profile of donor-specific production by the HLA-HI recipients was variable, with IL-2, IFN- γ and IL-10 being the main cytokines found (Figure 4.4b). In contrast to the cadaveric recipients, there was no significant correlation between the RRI and donor-specific IL-2, IL-4, IL-10 or IFN- γ production in the living-related cohort (Figure 4.5).

LRD recipients in both Groups A and B produced lower levels of all cytokines in response to donor stimulation compared with their CAD counterparts, probably reflecting the high proportion of HLA identical recipients. The lower production of donor-specific IL-2 in LRD recipients in Group A compared with those in Group B confirmed the results I found in the CAD recipients. However, the LRD recipients in Group A did not show the higher production of IL-4 seen in CAD recipients, nor was the significant correlations between the RRI and donor-specific IL-2, IL-4 or IFN- γ production which was seen in CAD recipients found in the LRD cohort.

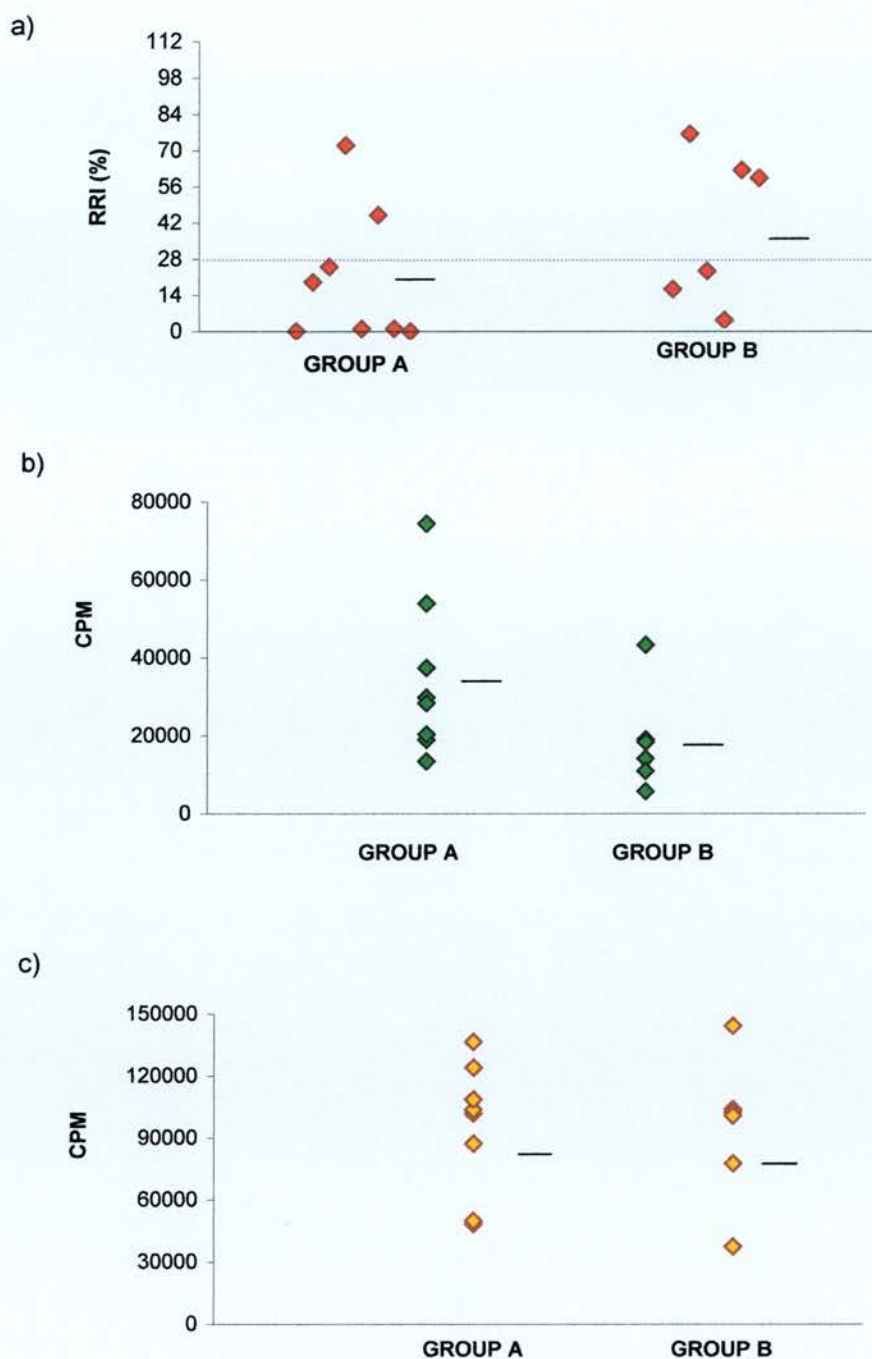


Figure 4.1: Proliferative responses of living-related recipients in Groups A and B. Results shown are the relative response indices (RRI) of each group, with DSH represented by the dotted line. (a), (b) and (c) show the mean ^3H -incorporation of triplicate cultures of recipient responder cells after stimulation with third party cells or PHA respectively. Group means are represented by the bars.

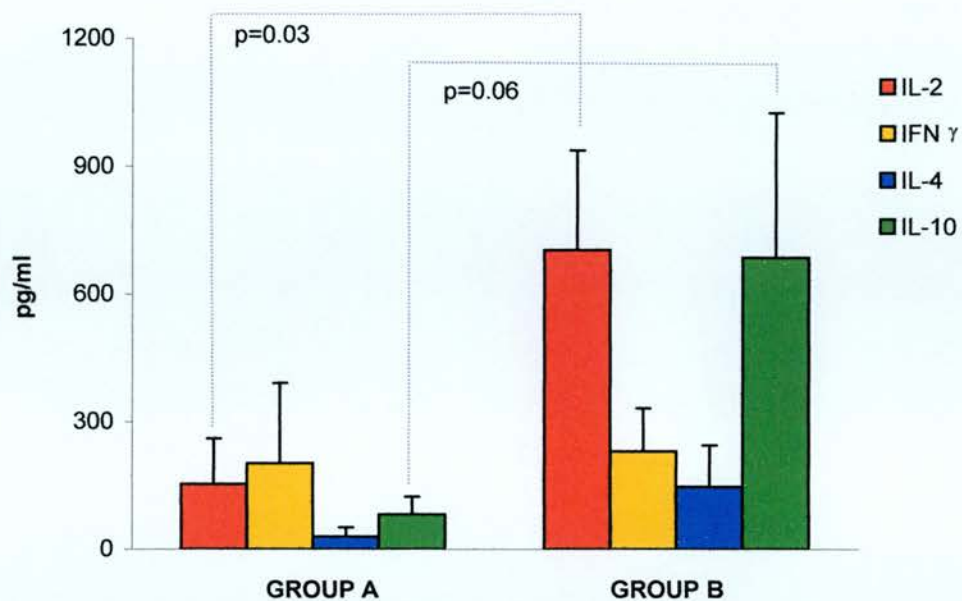


Figure 4.2: Cytokine production by patients in Group A (n=8) and Group B (n=6) in response to stimulation with donor cells. Results shown are mean donor-specific cytokine production \pm SEM after subtraction of the background responses in the presence of autologous stimulators.

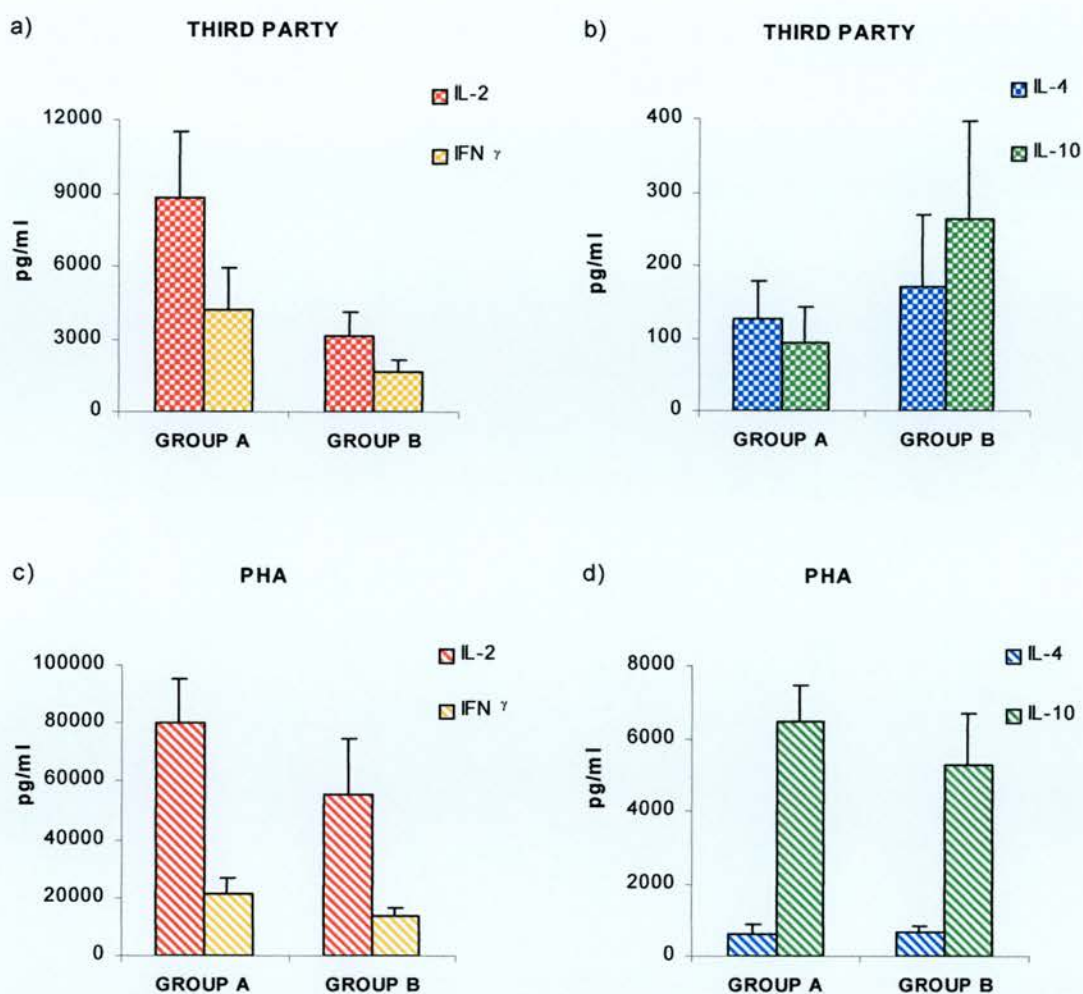


Figure 4.3: Cytokine production by living-related recipients in Group A (n=8) and Group B (n=6) in response to stimulation with third party stimulator cells (a and b) or PHA (c and d). Results shown are mean cytokine production \pm SEM after subtraction of the background responses in the presence of autologous stimulators.

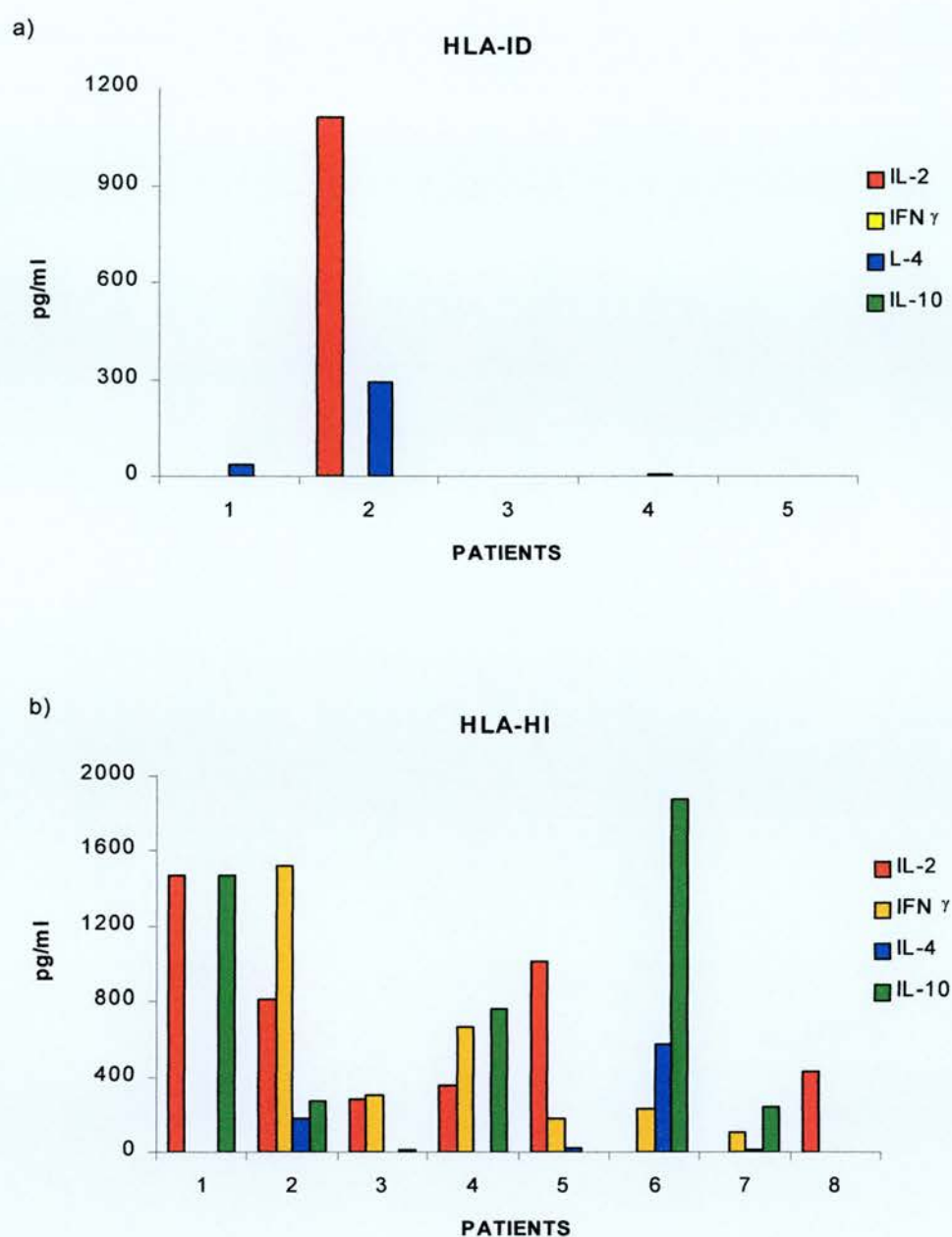


Figure 4.4: Cytokine production of HLA-identical (ID) (n=5) and HLA-haploidentical (HI) (n=8) living-related allograft recipients after stimulation with donor cells. Results shown are mean donor-specific cytokine production \pm SEM of triplicate cultures after subtraction of the background responses in the presence of autologous stimulators in HLA-identical (a) and HLA-haploidentical (b) recipients.

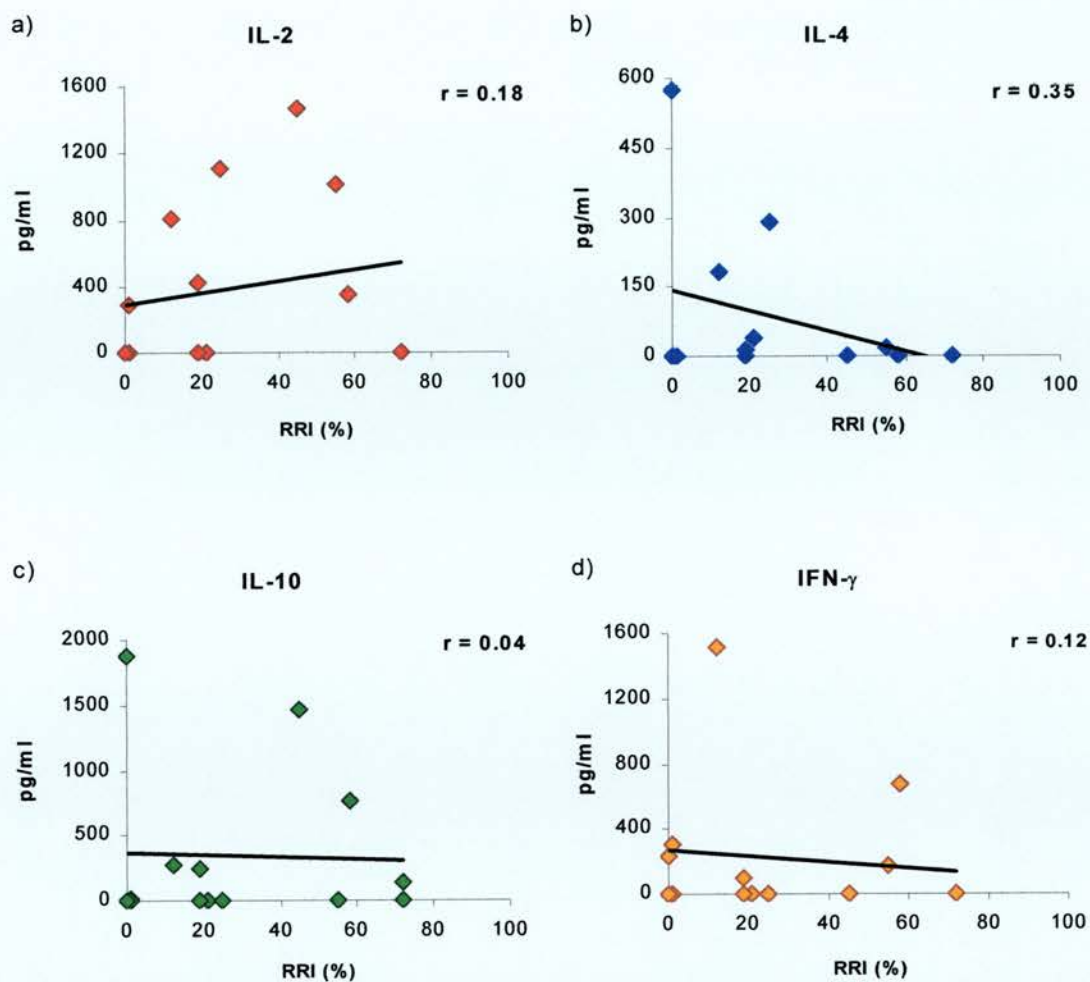


Figure 4.5: Correlation between the relative response index (RRI) and donor-specific cytokine production in living-related recipients.

TGF- β Production in Living-related Allograft Recipients

TGF- β was measured by ELISA in this cohort of patients as the non-quantitative PCR method I used to analyse TGF- β production in the cadaveric cohort showed that it was difficult to discriminate between cells stimulated with donor, autologous or third party cells in either Groups A or B.

LRD recipients in Group A produced lower levels of TGF- β protein than patients in Group B in response to stimulation with donor cells, but this was not statistically significant ($p=0.07$, Figure 4.6a). In contrast, Group A patients appeared to produce higher levels of TGF- β in response to third party stimulation (Figure 4.6b). The HLA-ID recipients produced lower levels of donor-specific TGF- β than the HLA-HI recipients, but this was not statistically significant ($p=0.13$, Figure 4.6c). Similar to the other cytokines analysed, there was also no correlation between the RRI and TGF- β production (Figure 4.7).

TGF- β production may be affected by the type of immunosuppression used and it may also influence the development of chronic rejection. I found that patients treated with tacrolimus ($n=4$) produced higher levels of donor-specific TGF- β than cyclosporin- treated patients ($n=6$), although this was not statistically significant ($p=0.22$, Figure 4.8a). In contrast, patients maintained on neither drug ($n=4$) produced almost undetectable levels of TGF- β , although when compared to the two other treatment groups, the differences did not reach statistical significance ($p=0.08$, Figure 4.8a). Additionally, I found that patients with biopsy-proven chronic rejection appeared to produce lower levels of TGF- β than patients without chronic rejection, although this was not statistically significant (Figure 4.8b).

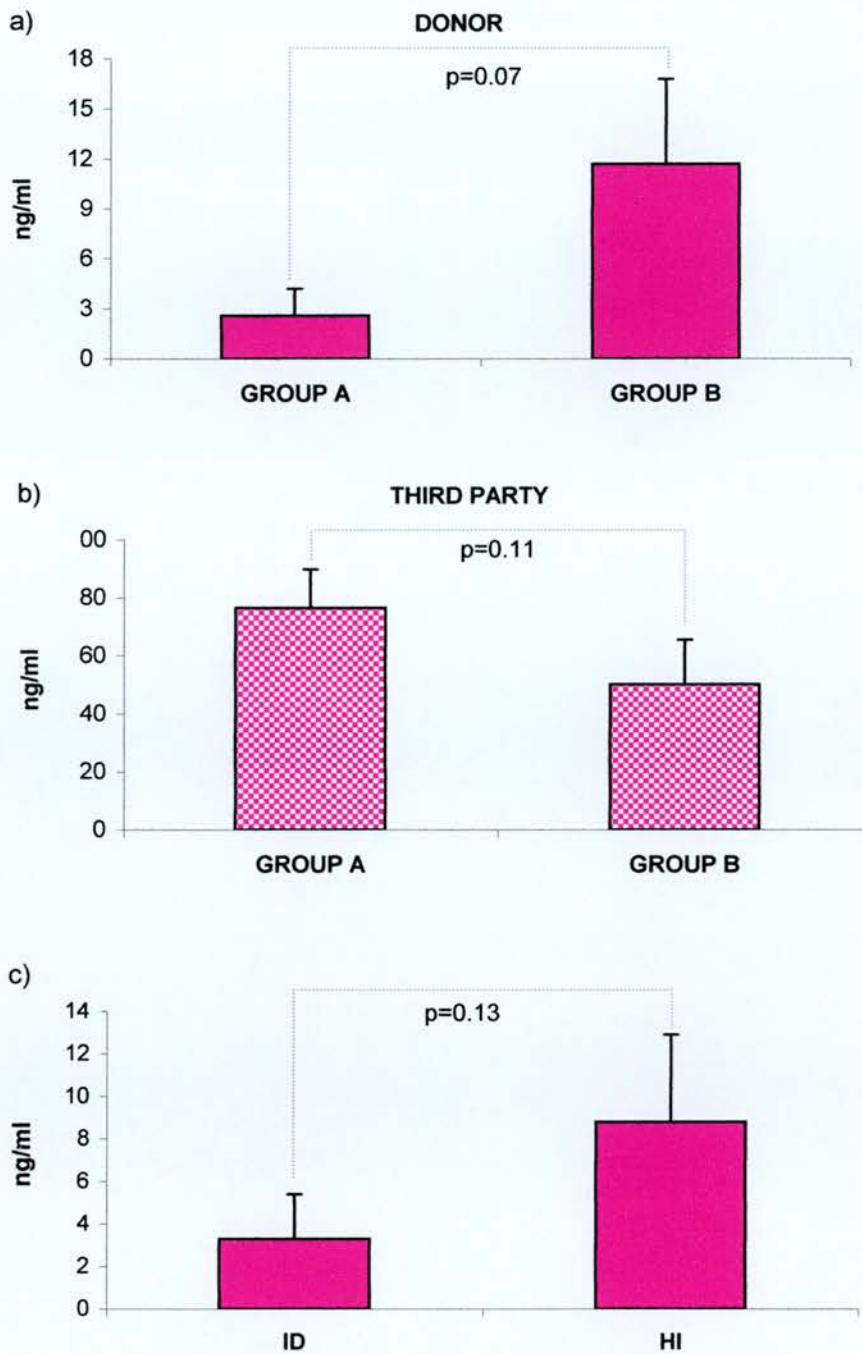


Figure 4.6: TGF- β production by living-related allograft recipients. TGF- β production in patients of Groups A (n=8) and B (n=6) in response to stimulation with donor cells (a) or third party cells (b). Donor-specific TGF- β production in HLA-identical (ID) (n=5) compared with haploidentical (HI) (n=8) recipients (c). Results shown are mean TGF- β production \pm SEM of triplicate cultures after subtraction of the background responses in the presence of autologous stimulators.

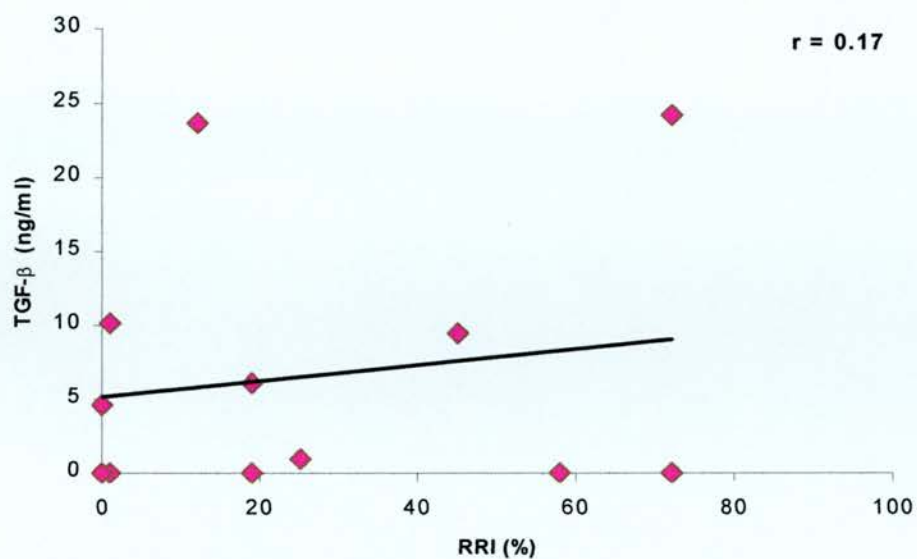


Figure 4.7: Correlation between the relative response index (RRI) and donor-specific TGF- β production in living-related allograft recipients (n=14).

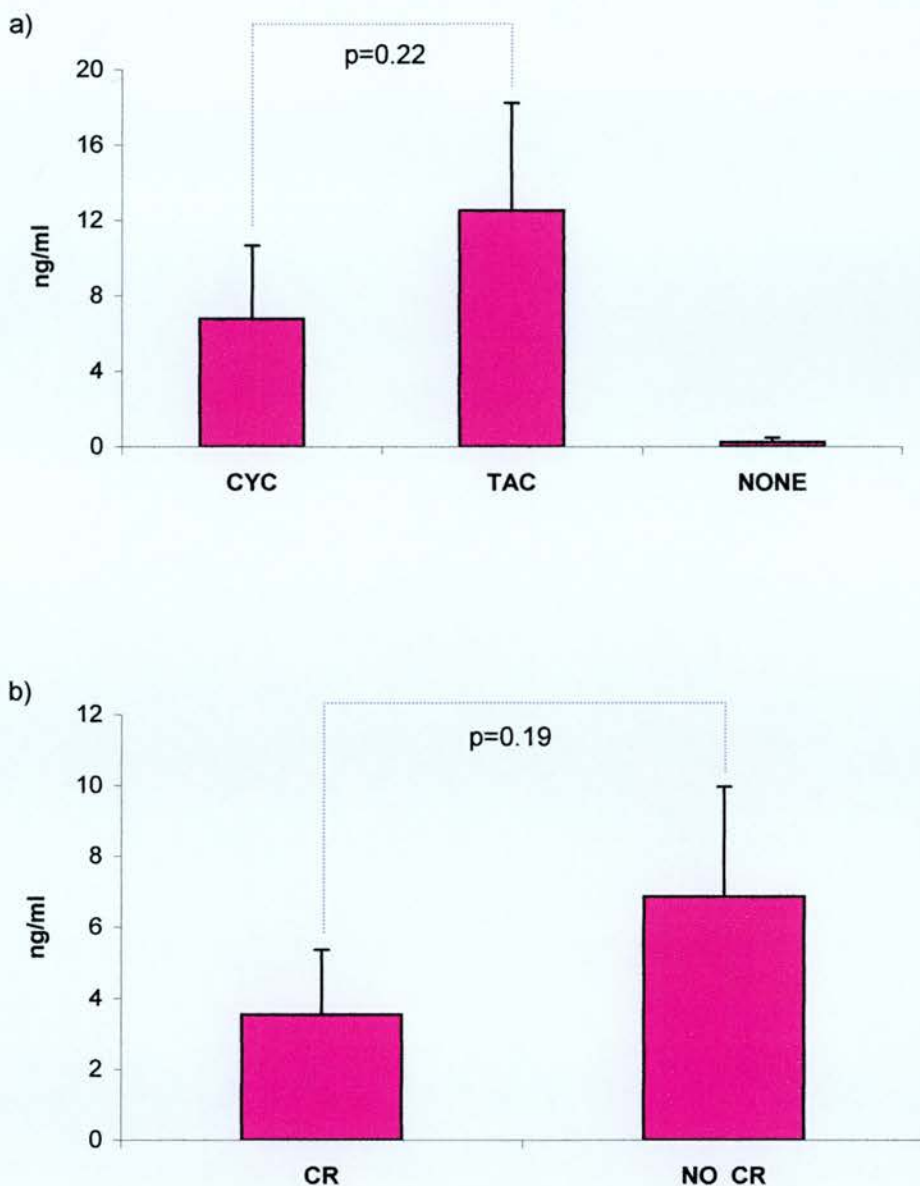


Figure 4.8: Donor-specific TGF- β production of patients maintained on different immunosuppressive regimens and of patients with chronic rejection. Results shown are mean TGF- β production \pm SEM after subtraction of the background responses in the presence of autologous stimulators of patients treated with cyclosporin (n=6), tacrolimus (n=4) or neither drug (n=4) (a), and of patients with biopsy-proven chronic rejection (CR, n=3) compared with those without (NO CR, n=11) (b).

EXPRESSION OF CYTOKINE mRNA

I had examined mRNA expression in only a small number of living-related recipients before I obtained the discordant results between PCR and ELISA analysis of cytokine production in the cadaveric recipients. At this point, I abandoned the PCR studies. Three patients were examined for cytokine mRNA assessment using RT-PCR, two from Group B and one from Group A (Figures 4.9).

IL-2

Donor-specific IL-2 mRNA was detected in all three patients including the patient in Group A in whom no measurable cytokine protein could be detected (GH, Figure 9). IL-2 mRNA was detected in third party and PHA stimulated cells in all the patients.

IFN- γ

Donor-specific IFN- γ gene expression and cytokine secretion was also detected in the two Group B patients (MH and CK, Figure 9). The Group A patient with good graft outcome did not secrete IFN- γ and no IFN- γ mRNA was detected. In response to stimulation with third party cells and PHA, IFN- γ protein was secreted and mRNA was detected by the Group B patients but the Group A patient showed no mRNA expression although IFN- γ protein was produced.

IL-4

The RT-PCR for IL-4 gene expression was unsuccessful.

IL-10

Both Group B patients had shown donor-specific IL-10 protein production and both had detectable donor-specific IL-10 expression of mRNA (MH and CK, Figure 9). Both these patients had poor graft outcome. The Group A patient did not show donor-specific gene expression or secretion of IL-10. All patients had IL-10 secretion and mRNA after third party stimulation, although it was weak in two patients (GH and MK). IL-10 mRNA was detected in all the PHA stimulated cells.

TGF- β

The Group B patient who produced high levels of TGF- β protein also had TGF- β mRNA expression (MH), while the other Group B patient did not have detectable protein or mRNA (CK, Figure 9). The Group A patient produced a low level of TGF- β protein and had mRNA expression (GH, Figure 9). In response to third party and PHA, TGF- β mRNA was detected and protein was secreted by the Group A patient (GH) and one Group B patient (MH), but no mRNA expression was detected in the other Group B patient (CK) when TGF- β protein was secreted.

There was a general correlation between donor-specific cytokine gene expression and protein secretion in this group, although in one patient (GH), IL-2 mRNA was detected when no cytokine protein was measured.

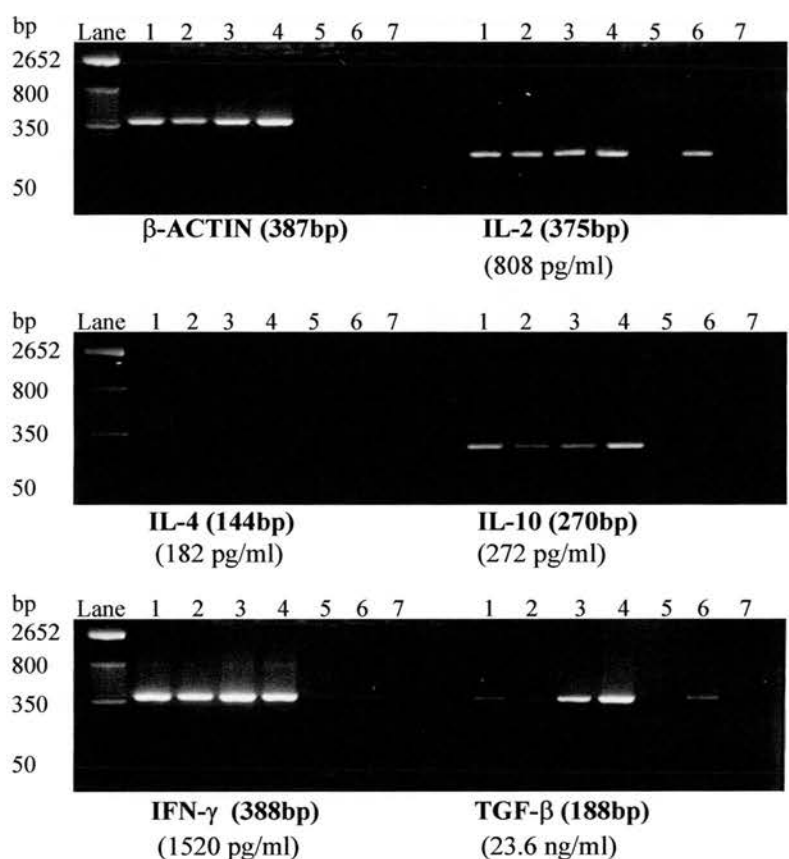


Figure 4.9a: MH

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Third Party
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Third Party
Lane 7: Neg Control

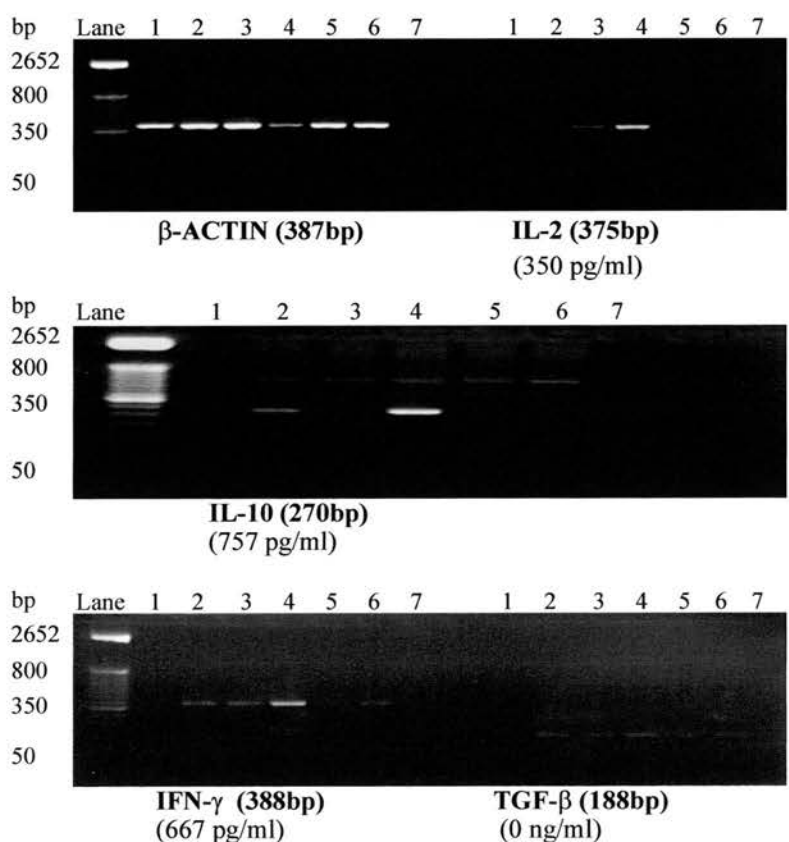


Figure 4.9b: CK

Lane 1: BGD
Lane 2: R/STIM
Lane 3: R/Third Party
Lane 4: R/PHA
Lane 5: STIM
Lane 6: Third Party
Lane 7: Neg Control

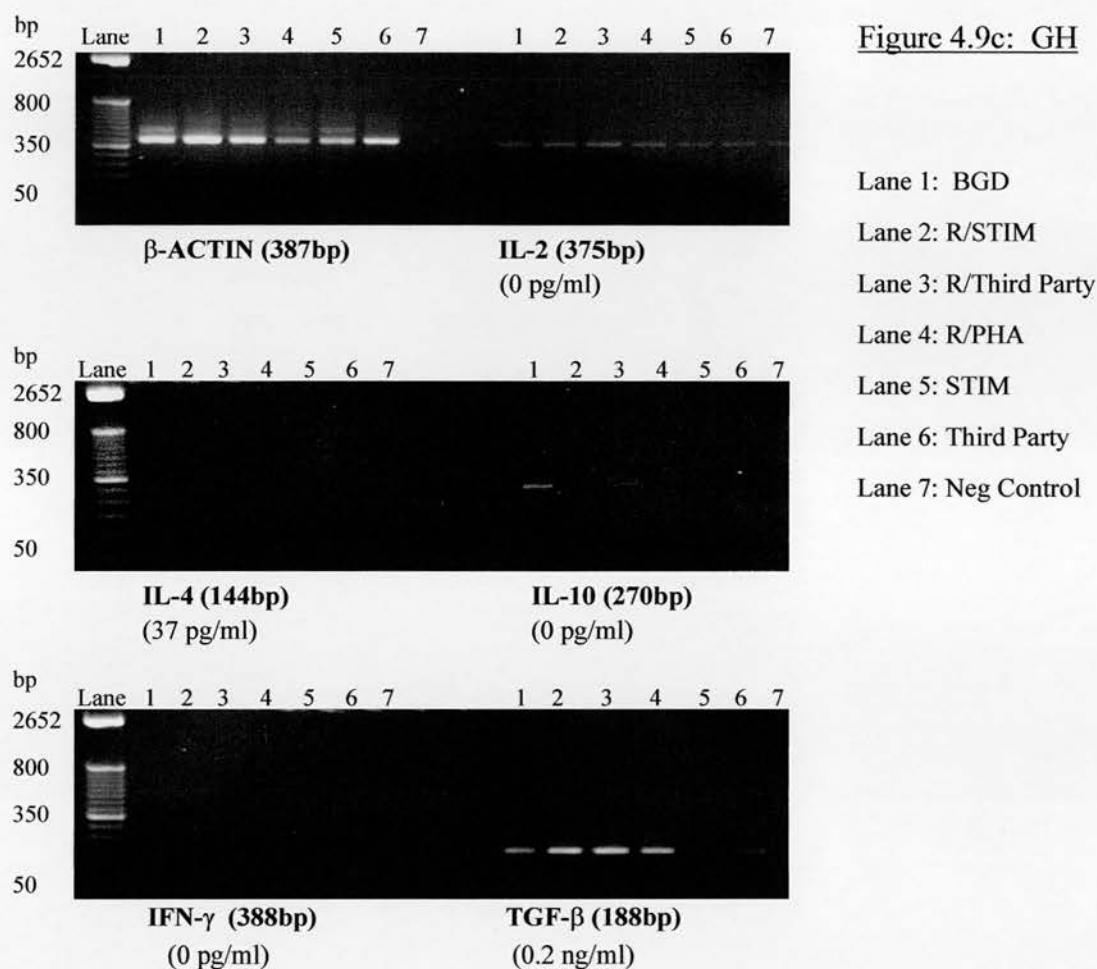


Figure 4.9 (a-c): IL-2, IL-4, IL-10, IFN- γ and TGF- β mRNA expression measured by RT-PCR in cells from patients from living related recipients. Lane 1 - autologous control (BGD), Lane 2 - donor-stimulated cells (R/S), Lane 3 - Third party stimulated cells (R/Pool), Lane 4 - PHA stimulated cells (R/PHA), Lane 5 - stimulator cells (STIM), Lane 6 - Third party cells (Pool), Lane 7 - negative control. The levels of donor-specific cytokine protein measured by ELISA in each patient are shown where appropriate. Insufficient stimulator cells were available for some patients.

CLINICAL FEATURES OF DSH

The clinical features of patients with and without DSH are compared in Tables 4.3 and 4.4. There was no significant difference in the median age of these groups (23.4 years versus 23.5 years) and although patients with DSH had been transplanted for a longer median duration than patients without DSH (7.7 years versus 5.3 years), this was not statistically significant (Table 4.3). DSH was found in all of the HLA-ID and 3/8 HLA-HI recipients (Table 4.3). The only HLA-NI recipient did not show DSH, but had a good graft outcome at 5 years post-transplant. The immunosuppressive regimens of patients who showed DSH in the LRD group was similar to those in the CAD group, with similar numbers of patients treated with cyclosporin, 75% versus 83% respectively. The incidence of DSH was also similar in Groups A (75% versus 89%) and B (50% versus 33%) in the LRD and CAD cohorts.

The level of renal function, as measured by serum creatinine, was similar in patients who showed DSH and those who did not (127 versus 116 $\mu\text{mol/L}$) (Table 4.3). Patients who showed DSH experienced significantly fewer acute rejection episodes than those who did not show DSH (44% versus 100%, $p=0.036$) (Table 4.3). DSH was detected in two patients with biopsy-proven chronic rejection, one of whom was an HLA-ID recipient, however overall there was no significant difference in the incidence of chronic rejection compared with patients who did not develop DSH (22% versus 20%). Six patients in the living-related cohort showed DSH and had good graft function (serum creatinine $< 140 \mu\text{mol/l}$).

Table 4.3: Clinical features of living-related recipients who showed DSH and those who did not.

GROUP	AGE TX (years)	TIME TX (years)	HLA-match			CREAT (μ mol/L)	AR	CR
			ID	HI	NI			
DSH (n=9)	23.4 (18.7 – 47.2)*	7.7 (4.1 – 21.4)	5	3	0	127 (97 – 354)	4	2
NON-DSH (n=5)	23.5 (16.7 – 33)	5.3 (4.5 – 9.9)	0	5	1	116 (86 – 257)	5	1
p value	0.21	0.11				0.80	0.036	0.89

AGE TX: age at transplant, TIME TX: time interval since transplant, CREAT: creatinine.

ID: identical; HI: haplo-identical; NI: non-identical.

AR: acute rejection, CR: chronic rejection.

*Results are shown in medians and ranges. (Wilcoxin signed rank test)

IMMUNOLOGICAL FEATURES OF DSH

Similar levels of donor-specific IL-2, IL-4, IFN- γ (Figure 4.10a) and TGF- β (Figure 4.10b) production were found in LRD patients with and without DSH. Although the DSH group appeared to produce less donor-specific IL-10 than patients who did not show DSH, this was not statistically significant ($p=0.38$, Figure 4.10a). Patients who showed DSH had similar proliferative responses following stimulation with third party (Figure 4.10c) and PHA (Figure 4.10d).

These findings contrast with the cadaveric cohort where patients with DSH produced significantly lower levels of IL-2 and higher levels of IL-4 compared with those who did not show DSH. Unlike the cadaveric cohort, a sub-group analysis of the ratio of donor-specific IL-4 and IL-2 production in relation to DSH and graft function could not be performed in the living-related cohort as the groups were all small.

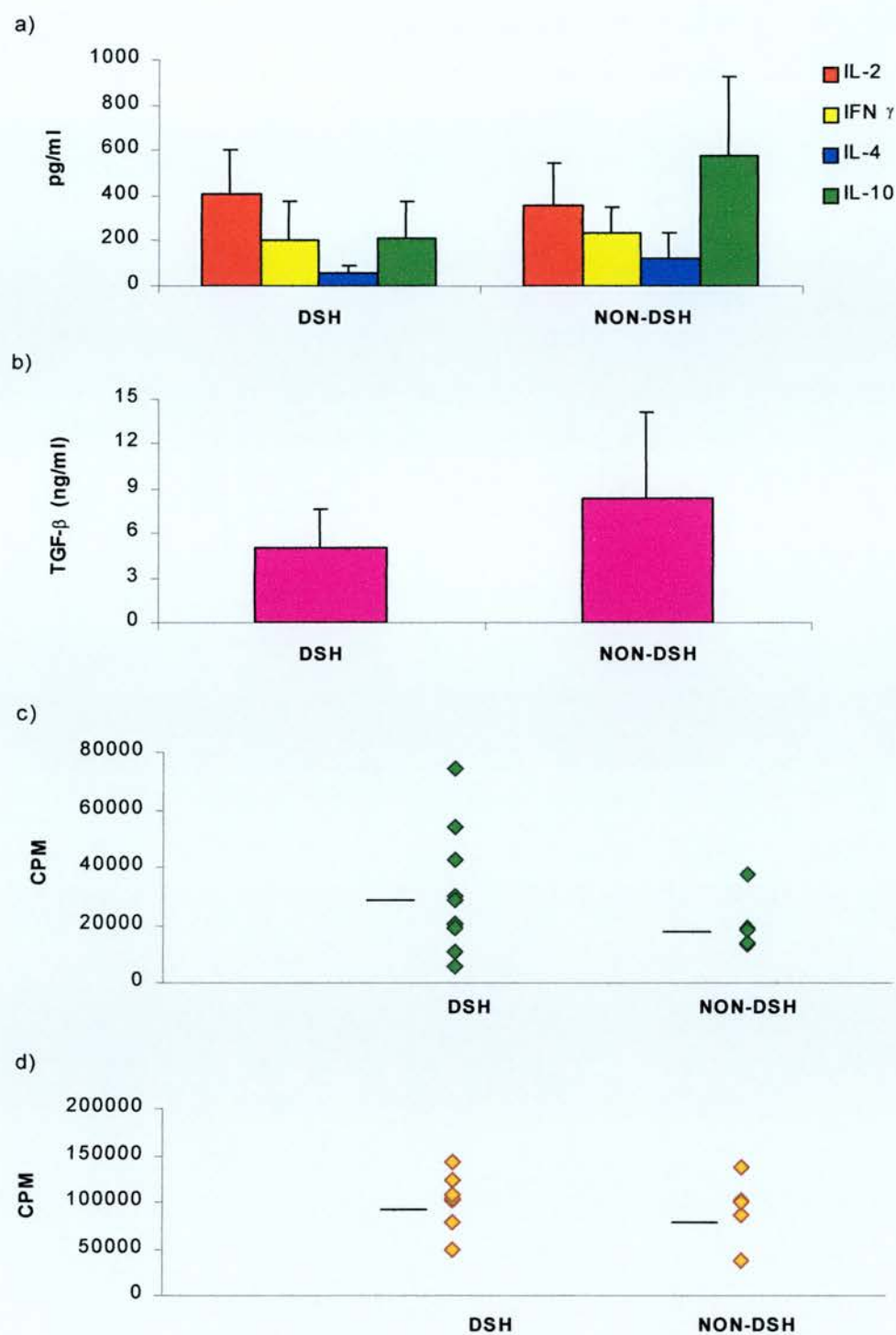


Figure 4.10: Immunological parameters of donor-specific hyporesponsiveness. Donor-specific cytokine production (a and b) and proliferative response to third party stimulator cells (c) or PHA (d) in patients who showed DSH (n=9) compared with those who did not (n=5). Results shown are mean concentrations of cytokines \pm SEM for the two groups after subtraction of the background responses in the presence of autologous stimulators (a and b), and the mean ^3H -TdR incorporation for triplicate cultures of individual recipient responder cells to stimulation with third party cells (c) and PHA (d). Group medians are shown by the bars.

SUMMARY

Living-related allograft recipients with a good graft outcome had significantly lower serum creatinine and produced significantly lower levels of donor-specific IL-2 than patients with a poor graft outcome. All HLA-identical recipients showed DSH and the level of donor-specific cytokine production appeared to be related to HLA-matching, as the HLA-identical recipients produced low levels of all cytokines. There was no significant difference in TGF- β production in patients maintained on cyclosporin or tacrolimus based immunosuppression and there was no correlation between donor-specific TGF- β production and chronic rejection.

In comparison to the cadaveric cohort, living-related recipients had similar graft function and acute rejection rate in the corresponding graft outcome groups. The incidence of DSH was also similar in the living-related (64%) and cadaveric (61%) cohorts and DSH was detected in patients with chronic rejection in both cohorts. Low donor-specific IL-2 production in patients with good graft outcome was a consistent finding in both cohorts. However, a few differences were seen between the living-related and cadaveric cohorts. Unlike the cadaveric cohort, there was no association between graft outcome and IL-4 production in the living-related cohort. A significant correlation was seen between RRI and IL-2, IL-4 and IFN- γ production in the cadaveric cohort, but there was also no correlation between RRI and production of all cytokines in the living-related cohort. Although DSH was associated with low donor-specific IL-2 production and high IL-4 production in cadaveric recipients, there was no association between DSH and cytokine production in the living-related cohort.

CHAPTER 5: MALIGNANCY COHORT

INTRODUCTION

A confounding factor in most immunological studies of transplant recipients is the effect of immunosuppression on the parameters which are to be measured. To try and estimate the possible effects of this in my study, I included a cohort of patients who had been maintained on low dose immunosuppression because they had developed a de novo solid organ malignancy post transplantation. By definition, these patients represent a group which have maintained good graft survival and a lower incidence of acute rejection. I hoped this group would allow me to assess better the significance of donor-specific hyporesponsiveness in transplant recipients given conventional immunosuppressive regimens. Additionally, donor-specific cytokine secretion has not been reported previously in patients of this kind.

Solid organ malignancy is often a fatal complication of transplantation with a short life expectancy after diagnosis. Indeed, the incidence of cancer among transplant recipients is estimated to be 3- to 4-fold greater than that of age-matched controls in the general population (Penn, 1998). Additionally, the risk of malignancy increases with the age of the recipient, type and duration of immunosuppression. As older patients are being considered for transplantation, more potent immunosuppressive agents are being used and graft survival has improved in the last two decades, it seems likely that the incidence of malignancy will increase in years to come. Therefore, it may become more important to identify patients at low risk of adverse immunological events in whom immunosuppression could be safely reduced.

PATIENTS

Ten first cadaveric graft recipients who developed a de-novo non-skin malignancy were entered in the study. Only a small number of patients was available for this study, as survival following diagnosis of malignancy was generally short. The tumours were 3 colo-rectal tumours, 1 gastric carcinoma with metastatic disease, 2 native renal tumours, 1 prostatic cancer, 1 non-small cell lung cancer and 1 patient with a tonsillar lymphoma. The age, duration since transplant, degree of HLA-match, serum creatinine, primary malignancy, and time to diagnosis of malignancy are shown for individual patients in Table 5.1. The median time to the diagnosis of malignancy following transplantation was 10.2 years (range 2.6-13.6 years) and only four patients were aged over 60 years at the time of diagnosis of malignancy. In this cohort, 9/10 patients have had a functioning graft for more than 10 years at the time of my study.

Immunosuppressive Regimens

Their immunosuppressive regimes at the time of recruitment were: prednisolone only (n=4), prednisolone and azathioprine (n=3), and prednisolone and cyclosporin (n=3). No patients were treated with OKT3 or polyclonal antibody therapy as part of the initial immunosuppressive regimen or for treatment of acute rejection.

Rejection History

There were two early and no late acute rejection episodes. None of the patients had chronic rejection. By comparison, none of the cadaveric (Chapter 3) or living-related (Chapter 4) recipients without malignancy were maintained on monotherapy; 69% of

the former and 29% of the latter received dual therapy; all other patients in these cohorts received triple therapy.

CLINICAL FEATURES

The median age of patients in the malignancy group was 40.8 years (range 26.3-61.9 years, Table 5.2) and median duration since transplant was 12.7 years (range 4.8-15.9 years, Table 5.2). The median degree of HLA-mismatch was 3 (range 1-5) and median serum creatinine was 105 $\mu\text{mol/L}$ (range 82-183 $\mu\text{mol/L}$). One patient had impaired renal function as a consequence of heart failure and presumed renal hypoperfusion. Four patients died with functioning grafts during the study.

When compared with patients in the cadaveric cohort without malignancy, there was no significant difference in age (40.8 years versus 39.1 years), the degree of HLA-mismatch (3 in both groups), or the time since transplantation (12.7 years versus 10.8 years, Table 5.2). In some clinical respects, the patients with malignancy were analogous to patients in Group A of the cadaveric cohort, as their median serum creatinine was 105 $\mu\text{mol/L}$, compared with 102 $\mu\text{mol/L}$ in Group A and 252 $\mu\text{mol/L}$ in Group B patients without malignancy. In addition, the acute rejection rate in patients with malignancy was 20%, compared with 22% in Group A and 66% in Group B non-malignancy patients.

Table 5.1: Clinical features and primary organ involvement of cadaveric recipients with malignancy.

PATIENT	AGE TX (years)	TIME TX (years)	HLA M/M	CREAT (μ mol/L)	I/SUPPRESSION	PRIMARY ORGAN	TIME DX (years)
1	26.3	14.1	5	121	PRED	COLON	11.1
2	49.2	15.9	3	104	PRED/ CYC	RENAL	10.8
3	42.6	15.9	1	98	PRED/ AZA	RENAL	12.8
4	37.8	13.7	3	183	PRED/ CYC	PROSTATE	9.8
5	54.6	11.7	3	106	PRED/ AZA	TONSILLAR	10.6
6	27.3	15.8	1	111	PRED	GASTRIC	13.6
7	31.7	11.3	1	91	PRED/ CYC	ANAL	9.1
8	39	10.3	1	82	PRED/ AZA	COLON	8.1
9	54.6	10	3	97	PRED	LUNG	9.8
10	61.9	4.8	3	109	PRED	UTERINE	2.6

AGE TX: Age at transplant, TIME TX: duration since transplant, M/M: degree of HLA-A, B and DR-mismatch, CREAT: creatinine, I/SUPPRESSION: immunosuppression (PRED- prednisolone, AZA- azathioprine, CYC- cyclosporin), TIME DX: time of diagnosis of malignancy.

Table 5.2: Clinical features of cadaveric recipients with malignancy compared with those without malignancy based on graft outcome.

GROUP	AGE TX (years)	TIME TX (years)	HLA M/M	CREAT ($\mu\text{mol/L}$)	TIME DX (years)	AR RATE	CR RATE
MALIGNANCY	40.8 (26.3 – 61.9)	12.7 (4.8 – 15.9)	3 (1 – 5)	105 (82 – 183)	10.2 (2.6 – 13.6)	20%	0
CAD -GROUP A	41.1 (11.3 – 57.8)	10.3 (7.6 – 17.5)	3 (0 – 5)	102 (56 – 132)	N/A	22%	0
CAD -GROUP B	34.6 (19.3 – 62)	9.6 (7.5 – 15.7)	3 (0 – 5)	252 (160 – 480)	N/A	66%	83%

AGE TX: age at time of transplant, TIME TX: duration since transplant, M/M: degree of HLA-A, B, and DR-mismatch, CREAT: creatinine, TIME DX: duration following transplant at the time of diagnosis of malignancy, AR: acute rejection, CR: chronic rejection.

CAD – GROUP A: good graft outcome, CAD – GROUP B: poor graft outcome.

*Results shown are the medians and ranges. N/A: not applicable.

DONOR-SPECIFIC PROLIFERATIVE RESPONSES IN VITRO

The mean RRI in the malignancy cohort was $21.3 \pm 7.2\%$ compared with $14.7 \pm 3.2\%$ in Group A and $36.7 \pm 5.8\%$ in Group B of the non-malignancy cadaveric cohort (Figure 5.1a). Donor-specific hyporesponsiveness, as defined by an RRI $\leq 28\%$, was detected in 9/10 (90%) patients with malignancy, compared with 22/36 (61%) of cadaveric recipients without malignancy overall, but this difference was not statistically significant. The incidence of DSH in the malignancy cohort was identical to that of non-malignancy patients with similar graft outcome in Group A (90% versus 89%) and was much higher than that of the patients with poor graft outcome in Group B (90% versus 33%). Proliferative responses to third party cells (Figure 5.1b) and PHA (Figure 5.1c) in the malignancy group were comparable to those in the non-malignancy cadaveric cohort, suggesting that there was no generalised immunosuppression.

PRODUCTION OF CYTOKINES

Lymphocytes from patients with malignancy produced low levels of IL-2, IL-4 and IFN- γ , but produced high levels of IL-10 when stimulated with donor cells (Figure 5.2a). The mean level of IL-10 produced by these patients was approximately four-fold higher than any other cytokine produced and twice that found in the Group A patients without malignancy who shared good graft outcome, although this was not statistically significant. In Chapter 3, I found that low IL-2 and high IL-4 production was associated with good graft outcome in patients without malignancy. However,

the patients with malignancy produced low levels of both IL-2 and IL-4, despite their good graft outcome.

Compared with the cadaveric recipients in Group B who had a poor graft outcome, the malignancy group produced similar levels of IL-10, lower levels of IFN- γ and significantly lower levels of IL-2 ($p=0.009$, Figure 5.2a). In response to third party stimulation, patients with malignancy produced higher levels of IL-2 and IFN- γ and similar levels of IL-4 and IL-10 compared with both sub-groups of patients without malignancy (Figure 5.2b). The level of IL-2 produced by the malignancy group was lower than the non-malignancy sub-groups following stimulation with PHA (Figure 5.2c), but similar levels of IL-4, IL-10 and IFN- γ were produced.

When all patients with malignancy were considered together, they produced a lower mean level of IL-2 in response to donor stimulation than patients without malignancy, but there were no significant differences in donor-specific cytokine production between the two groups (Figure 5.3a). The patients with malignancy who showed DSH produced higher levels of IL-2 than their counterparts without malignancy, although they produced significantly lower levels when compared with the Group B non-malignancy patients who did not have DSH ($p=0.03$, Figure 5.3b). However, the malignancy group with DSH produced lower levels of IL-4 compared with their non-malignancy counterpart and similar levels compared with patients without DSH, although these differences were not significant (Figure 5.3b). PCR studies were not performed in this patient cohort as discordant results were obtained from the cadaveric and living-related groups.

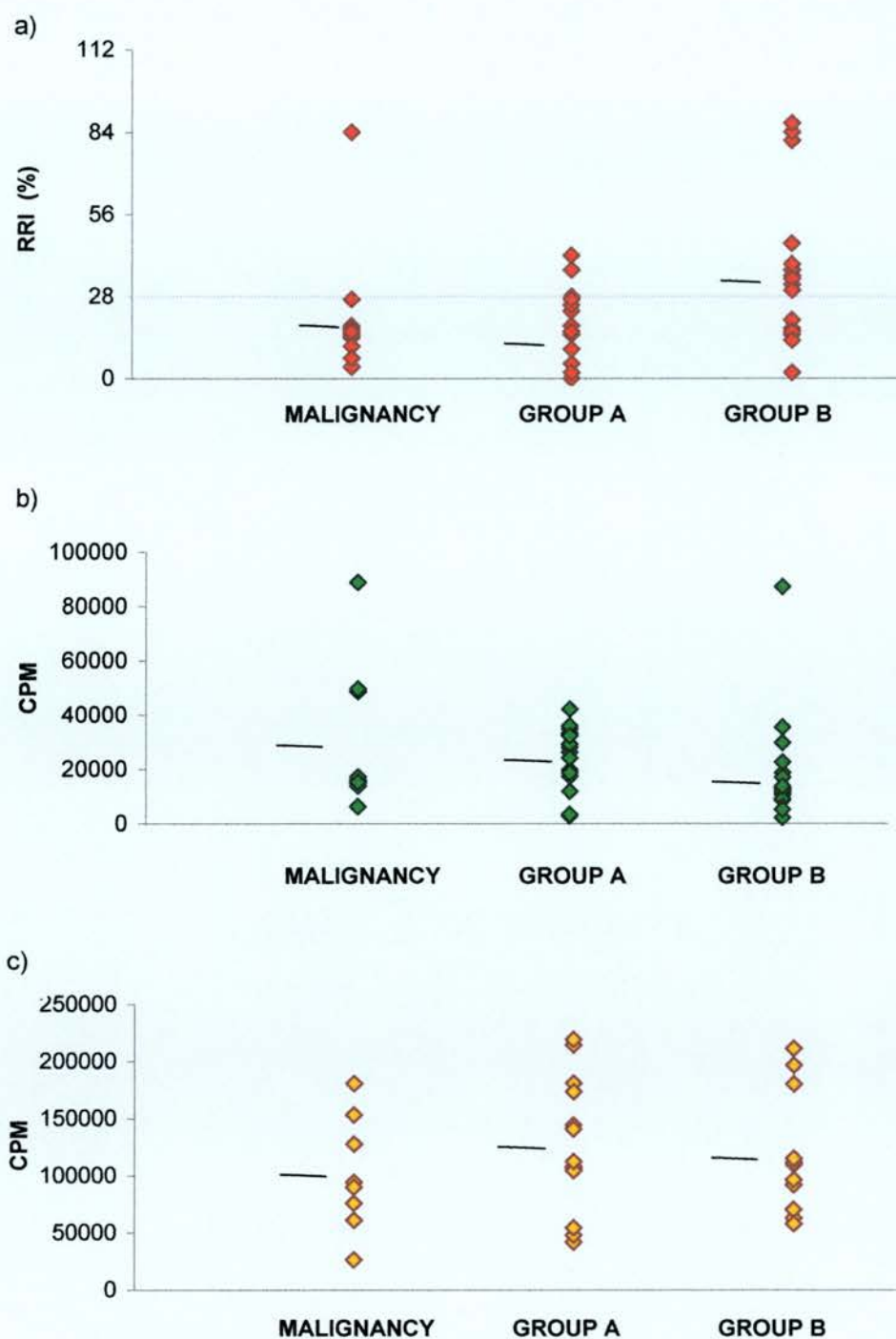


Figure 5.1: Proliferative responses of cadaveric recipients with malignancy compared with patients in Groups A and B without malignancy. Results shown are the relative response indices (RRI) of each group with DSH represented by the dotted line (a). (b) and (c) show the mean ^3H -TdR incorporation of triplicate cultures of recipient cells after stimulation with third party cells or PHA respectively. Group means are represented by the bars, * $p < 0.05$.

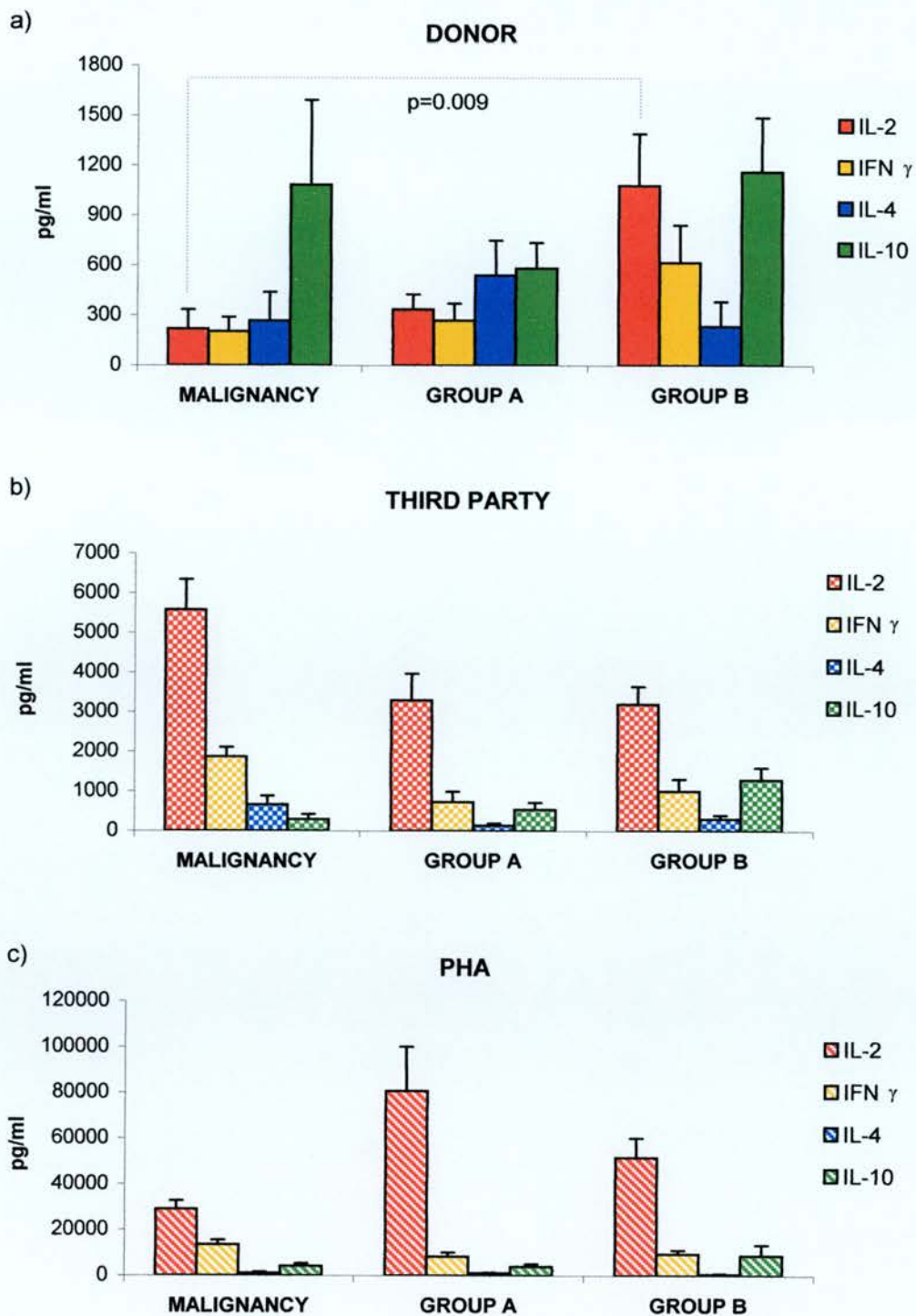


Figure 5.2: Cytokine production of cadaveric recipients with malignancy (n=10) compared with patients without malignancy in Groups A (n=18) and Group B (n=18). Results shown are mean donor-specific cytokine production \pm SEM in response to stimulation with donor cells (a), third party cells (b) and PHA (c) after subtraction of background responses in the presence of autologous stimulators.

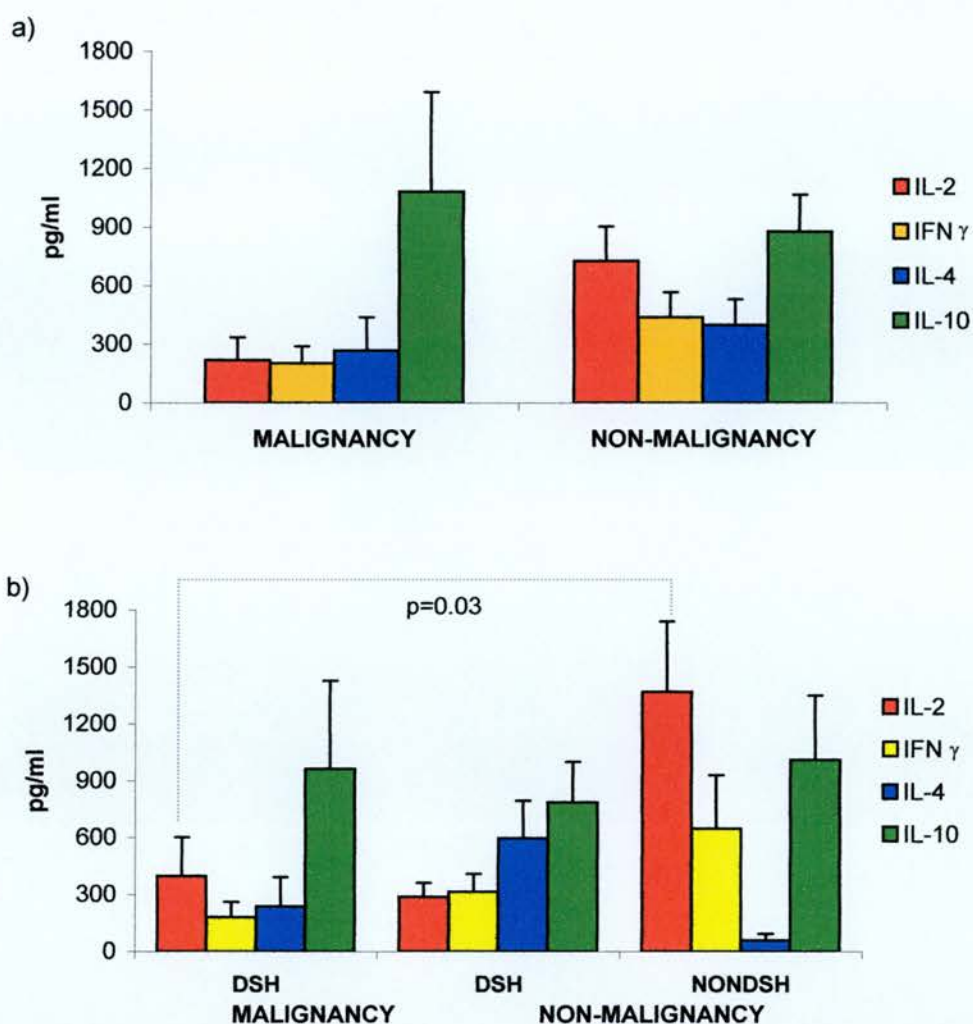


Figure 5.3: Donor-specific cytokine production of cadaveric recipients with malignancy compared with patients without malignancy. Results shown are mean donor-specific cytokine production \pm SEM after subtraction of background responses in the presence autologous stimulators in patients with (n=10) compared with those without malignancy overall (n=36) (a), and in patients with malignancy who showed DSH (n=9) compared with those without malignancy who showed DSH (n=22) or did not (n=14) (b).

SUMMARY

The majority of the cadaveric recipients who developed a solid organ malignancy as a complication post-transplantation shared some of the clinical and immunological features I found in cadaveric recipients with good graft function that did not have malignancy. These included good graft function, low acute rejection rate and DSH, as measured by proliferation and low IL-2 production. Low IL-2 production was also a consistent finding among living-related recipients with good graft outcome. However, patients with malignancy appeared to produce more IL-10 and less IL-4 than non-malignancy cadaveric recipients with good allograft outcome. As the patients in this study had no consistent evidence of generalised immunosuppression, these results support the idea that a state of donor-specific hyporesponsiveness and low donor-specific IL-2 production may partly explain their better graft outcome. The other aim of my study was to determine whether donor-specific hyporesponsiveness was associated with a particular pattern of cytokines, and my data suggests that cadaveric recipients with or without malignancy who developed DSH consistently showed low IL-2 production.

CHAPTER 6: PROSPECTIVE STUDY

INTRODUCTION

There have been many reports of donor-specific hyporesponsiveness (DSH) developing in renal allograft recipients following transplantation, but the production of cytokines has never been correlated with the evolving DSH. Therefore, in this arm of the study, I performed a prospective study to follow donor responsiveness during the first year post-transplant and to determine whether patients who developed DSH produced a particular pattern of cytokines. In Chapter 3, I showed that DSH was associated with a favourable graft outcome and low donor-specific IL-2 and high IL-4 production in long-term cadaveric graft survivors. If a similar cytokine profile occurs in patients who develop DSH and maintain good graft function at the end of the first year post-transplant, it could be a useful guide to tailoring immunosuppression.

This chapter will describe the clinical and immunological features of cadaveric and living-donor renal allografts recipients during their first year post-transplant. The clinical course of all patients was followed for a further year to assess graft outcome and to examine for the development of chronic rejection, which is not commonly detected in the first year post-transplant. Patients were analysed for donor responsiveness immediately before and at three, six and twelve months post-transplant using mixed lymphocyte cultures, cytokine production and cytokine gene expression as before.

PATIENTS

All recipients of first cadaveric or live-donor renal allografts were eligible for this arm of the study. The exclusion criteria were pregnancy and inability to give informed consent. Most patients received one or more blood transfusions during the course of renal replacement therapy prior to transplantation, as treatment for anaemia and not as part of a planned pre-transplant blood transfusion programme. The complement-dependent cytotoxicity crossmatch was negative before transplantation in all patients.

CADAVERIC RECIPIENTS

Twenty-two adult recipients (17 male and 5 female) of cadaveric (CAD) renal transplants performed between October 1998 and June 1999 were entered into the study. Eight patients could not be analysed: two due to early graft failure, both secondary to severe acute rejection; one withdrew from the study at 9 months follow-up; three had poor recipient cell number and viability; and two had inadequate donor cells. Therefore only 14 patients completed 12 months follow-up and were available for analysis. The median degree of HLA-mismatch was 2 (range 0-6). The aetiology of renal failure was: chronic glomerulonephritis (n=4), polycystic kidney disease (n=3), chronic pyelonephritis (n=1), interstitial nephritis (n=1), Wegener's granulomatosis (n=1), and unknown (n=4).

All patients received methylprednisolone 1g intravenously immediately pre-transplantation, thereafter conventional triple therapy consisting of prednisolone, azathioprine and cyclosporin was given as maintenance therapy. Five patients experienced acute rejection, of which four occurred early and one late (Table 6.1).

Two patients developed chronic rejection and graft function declined. The immunosuppressive regimen was modified in these seven patients as a result of acute or chronic rejection, with four patients being converted from azathioprine to mycophenolate, and three patients being converted from cyclosporin to tacrolimus.

LIVING-DONOR RECIPIENTS

Six adult recipients (all male) of living-donor (LD) renal allografts, performed between October 1998 and May 1999 were entered into the study. Two patients could not be analysed due to early graft failure: one due to steroid-resistant acute rejection and the other as a result of renal vein thrombosis. The remaining four patients all completed 12 months follow-up. With respect to HLA-matching: one patient was HLA-identical (ID), two were HLA-haploidentical (HI), and one patient who received a graft from his spouse was HLA-nonidentical (NI). The aetiology of renal failure in these patients was: chronic glomerulonephritis (n=1), diabetic nephropathy (n=1), congenital renal dysplasia (n=2).

All patients received methylprednisolone 1g immediately pre-transplantation. Thereafter, conventional triple therapy consisting of prednisolone, azathioprine and cyclosporin was given as maintenance therapy. Three patients experienced acute rejection – two occurred early, and one late (Table 6.1). The immunosuppressive regimen was modified in these patients, with all being converted to tacrolimus from cyclosporin following acute rejection. The HLA-ID recipient did not experience acute rejection and none of the patients have developed chronic rejection.

Table 6.1: Clinical features of patients followed prospectively for one year.

GROUP	AGE TX (yrs)	M/M	PRA (%)	AR		CR
				EARLY	LATE	
CAD (n=14)	47.9 (24.6 - 64)	2 (0 - 6)	2 (0 - 32)	4	1	2
LD (n=4)	33.7 (18.4 - 41.9)	ID-1 HI-2 NI-1	4 (0 - 66)	2	1	0

AGE TX: age at transplant, M/M: degree of HLA-A, B and DR-mismatch, PRA: panel reactive antibody level, AR: acute rejection, CR: chronic rejection.

CAD: cadaveric, LD: living-donor.

*Results are shown as medians and ranges where appropriate.

Table 6.2: Renal function over the first 12 months post-transplant.

GROUP	CREATININE (μmol/L)			
	Pre-Tx	3 months	6 months	12 months
CAD (n=14)	918 (505 - 1079)	157 (71 - 217)	137 (83- 284)	130 (95 - 280)
LD (n=4)	762 (501 - 1068)	134 (117 - 174)	120 (102 - 143)	144 (125 - 174)

Pre-Tx: pre-transplant

CAD: cadaveric, LD: living-donor.

*Results are shown as medians and ranges.

CLINICAL FEATURES

The median age of the CAD recipients was 47.9 years (range 24.6-64 years) and 33.7 years (18.4-41.9 years) for the LD recipients. This difference did not reach statistical significance ($p=0.07$, Table 6.1). There was also no significant difference in the degree of sensitisation to HLA-antigens as measured by the panel reactive antibody (PRA) level, or in serum creatinine levels pre-transplant and at 3, 6 and 12 months post-transplant between CAD and LD recipients (Table 6.2). The baseline immunosuppressive regimen was similar in both groups of patients, with all patients receiving conventional triple therapy irrespective of HLA-matching and PRA status. Nevertheless, the incidence of acute rejection was higher among the LD group than the CAD group (75% versus 36%), although this difference was not statistically significant. The level of graft function in CAD and LD recipients was monitored for 24 months to assess clinical stability. Two patients experienced late acute rejection, one CAD recipient at 5 months post-transplant (Figure 6.1a) and one LD recipient at 18 months post-transplant (Figure 6.1b). The level of serum creatinine did not return to its previous baseline in either patient. Two CAD recipients who showed persistently sub-optimal graft function were found to have biopsy-proven chronic rejection (Figure 6.1a).

Nine CAD recipients fitted the criteria for good graft outcome defined by Group A in Chapter 3 as having good allograft function (creatinine $< 140 \mu\text{mol/l}$) with either no, or early steroid responsive acute rejection. The remaining five patients had impaired graft function one year post-transplant and three of these met the criteria for poor graft outcome, as defined by Group B in Chapter 3 (one developed late acute rejection and two developed chronic rejection). Two LD

recipients had good graft function despite early steroid-responsive acute rejection and met the clinical criteria for Group A. One patient who experienced late acute rejection had impaired graft function and met the criteria for Group B. The HLA-ID patient had no rejection episodes, but had impaired graft function attributable to obstructive uropathy and required nephrostomy and urological intervention.

DONOR-SPECIFIC PROLIFERATIVE RESPONSES IN VITRO

Recipient (responder) lymphocytes were obtained from blood taken immediately before transplantation and thereafter at 3, 6 and 12 months post-transplant, always before the morning dose of cyclosporin or tacrolimus at a routine clinic visit. Donor (stimulator) cells were isolated by the local Tissue Typing Laboratory and stored frozen until ready for use. As in Chapter 3, the capacity of donor cells to induce proliferation by normal responder cells was tested to ensure their viability. Patients were excluded from the analysis if the donor or recipient cell viability was poor, cell count too low, or if the donor cells were unable to induce proliferation in a normal control. As control stimulator cells, the pool of cells from five normal volunteers was used as in Chapter 3. All the samples from all the time points from each patient were analysed at the same time to avoid day to day variation in the assays.

The mean relative response index (RRI) immediately pre-transplant in the CAD group was 63.4% compared with 145.7% (Table 6.3) in the LD group. Three cadaveric and one living-donor recipient had a $RRI \leq 28\%$ prior to transplantation, but did not all show DSH at one year post-transplant. Nevertheless, these patients experienced no acute rejection, as they were well-matched to their donors. Although the RRI fluctuated in most patients through-out the study period, at 12 months the

mean RRI in each group was identical to that found pre-transplant (60.1% and 139.1% in the CAD and LD groups respectively). Notably, the HLA-NI recipient experienced early acute rejection and remained strongly responsive to donor cells through-out the time course with a RRI of 345.8% pre-transplant and 374.7% at 12 months post-transplant.

Table 6.3: RRI over the first 12 months post-transplant.

GROUP	RRI (%)			
	Pre-Tx	3 months	6 months	12 months
CAD (n=14)	63.4 ± 11.6	73.1 ± 14.9	67.1 ± 19.2	60.1 ± 14
LD (n=4)	145.7 ± 71.7	178.5 ± 133.4	126.7 ± 50.9	139.1 ± 81.6
p value	0.34	0.49	0.36	0.41

RRI: relative response index, Pre-Tx: pre-transplant.

CAD: cadaveric, LD: living-donor (LD)

*Results are shown as means and SEM. (Wilcoxin signed rank test).

Among the cadaveric recipients, DSH defined as an RRI \leq 28%, was detected in 3/14 (21%) patients at 3 months post-transplant, in 4/14 (29%) patients at 6 months post-transplant and in 5/14 (36%) recipients at 12 months post-transplant (Figure 6.2a). At the end of the follow-up period, DSH was found in only 3 of the 9 cadaveric recipients who met the clinical criteria for good graft outcome and in one patient who developed chronic rejection. The remaining cadaveric recipient who

showed DSH had impaired graft function, but no history of acute or chronic rejection. Among the 4 LD recipients, DSH was detected only in the patient who received an HLA-ID graft and showed an RRI $\leq 28\%$ throughout the first year post-transplant (Figure 6.2b). Although this patient had no acute rejection, graft function was impaired and therefore did not meet the clinical criteria for good graft outcome. All patients showed good proliferative responses after stimulation with third party cells (Figures 6.3a,c) or PHA (Figures 6.3b,d), with no significant differences in these responses throughout the study period. Therefore, as I found in the retrospective study described in Chapter 3, DSH was not confined to patients with good allograft outcome and was not a result of general immune suppression.

Therefore, at 12 months post-transplant DSH was detected in 6/18 (33%) of the patients. The level of donor responsiveness fell from the pre-transplant level in all six patients who showed DSH at one year (Figure 6.4a). Of the twelve patients who did not show DSH at one year post-transplant, the RRI rose in 3 patients (GM, KM, and JR), remained virtually unchanged in 6 patients (AC, DM, GO, JH, JMc, and JB) and fell in only 3 patients (GH, GD, JA) (Figure 6.4b). In addition, two of these patients (KM and JR) had an RRI $\leq 28\%$ pre-transplant, but did not show DSH at 12 months post-transplant. There was no significant difference in the pre-transplant RRI between patients who developed DSH and those who did not, but there was a significant difference in RRI between these patients at 3 ($p=0.03$), 6 ($p=0.005$) and 12 ($p=0.004$) months post-transplant (Figure 6.4c). DSH was not due to general immune suppression, as the proliferative responses to third party cells (Figure 6.5a and c) and PHA (Figures 6.5b and 6.5d) were similar in patients who showed DSH and those who did not.

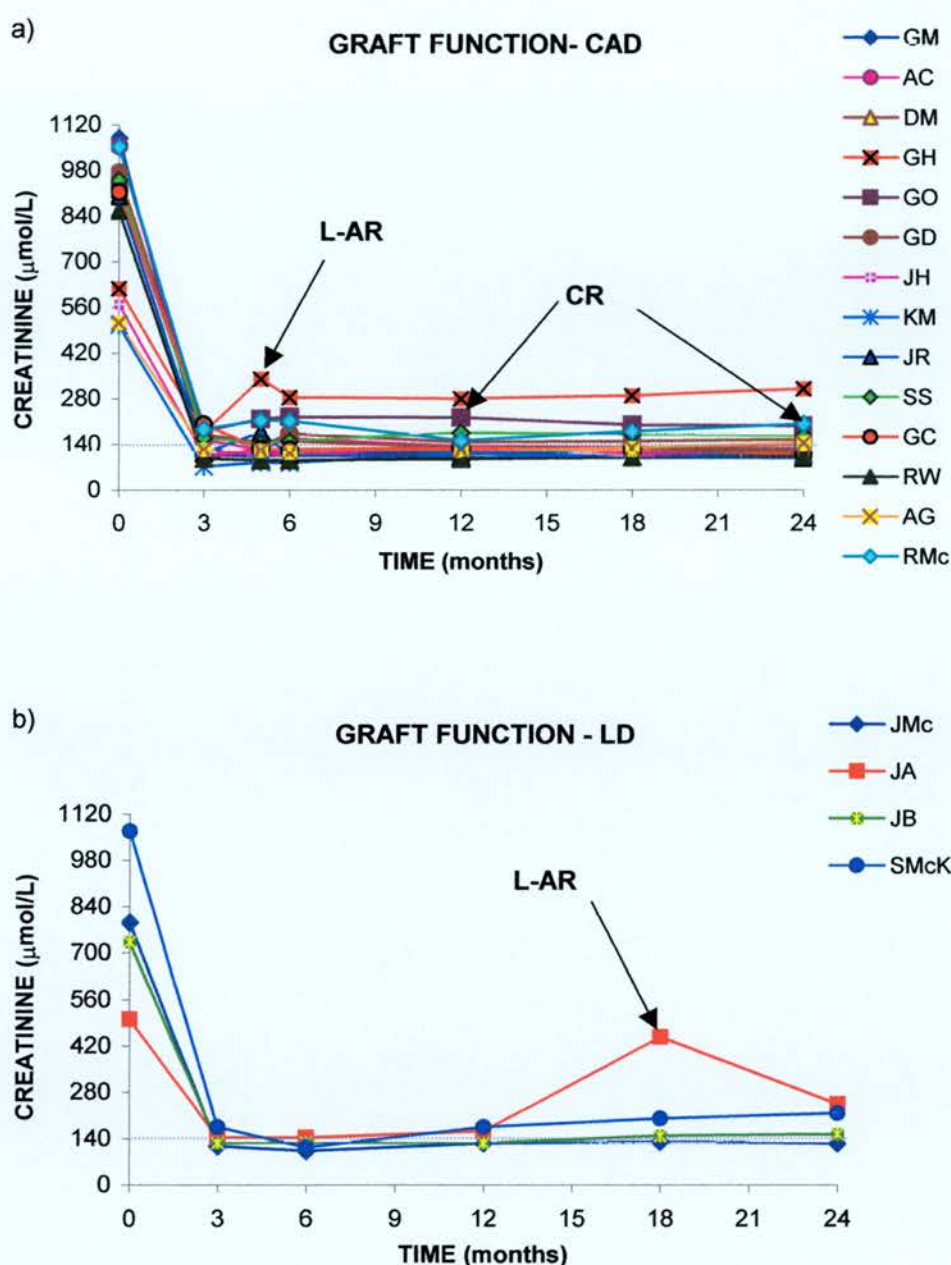


Figure 6.1: Graft function over the first two years post-transplant of renal allograft recipients followed prospectively. Results shown are the serum creatinine of cadaveric (CAD, $n=14$) (a) and living-donor (LD, $n=4$) (b) recipients over the first 24 months post-transplant, where the dotted line represents good graft function as defined by serum creatinine <140 mmol/L. Episodes of late acute rejection (L-AR) and chronic rejection (CR) are shown by the arrows.

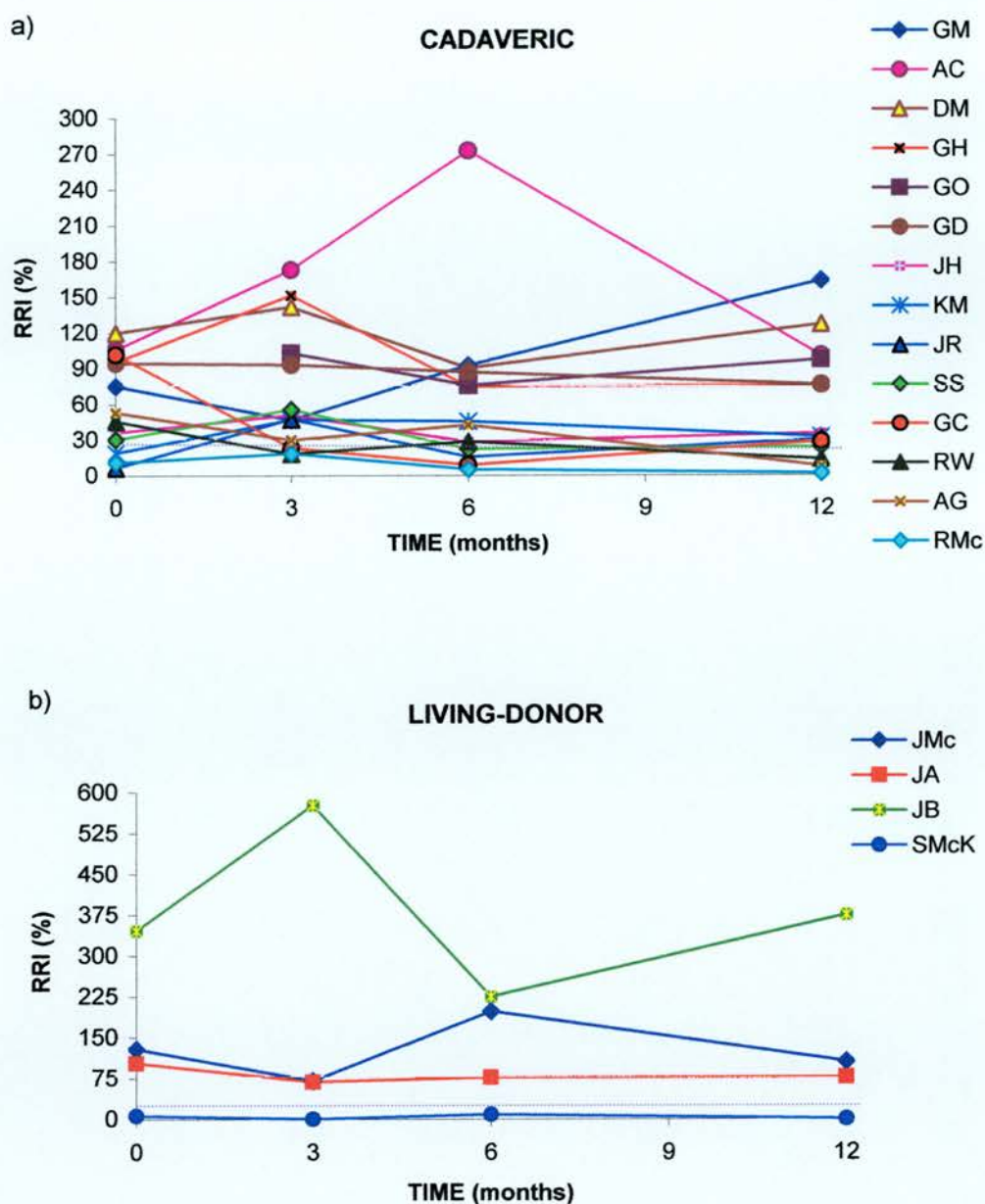


Figure 6.2: Relative response indices (RRI) of individual cadaveric (a) and living-donor (b) recipients over the first 12 months post-transplant. Results shown are the RRI of cadaveric ($n=14$) (a) and living-donor ($n=4$) (b) recipients over the first 12 months post-transplant. DSH, defined as $RRI \leq 28\%$, is represented by the dotted line.

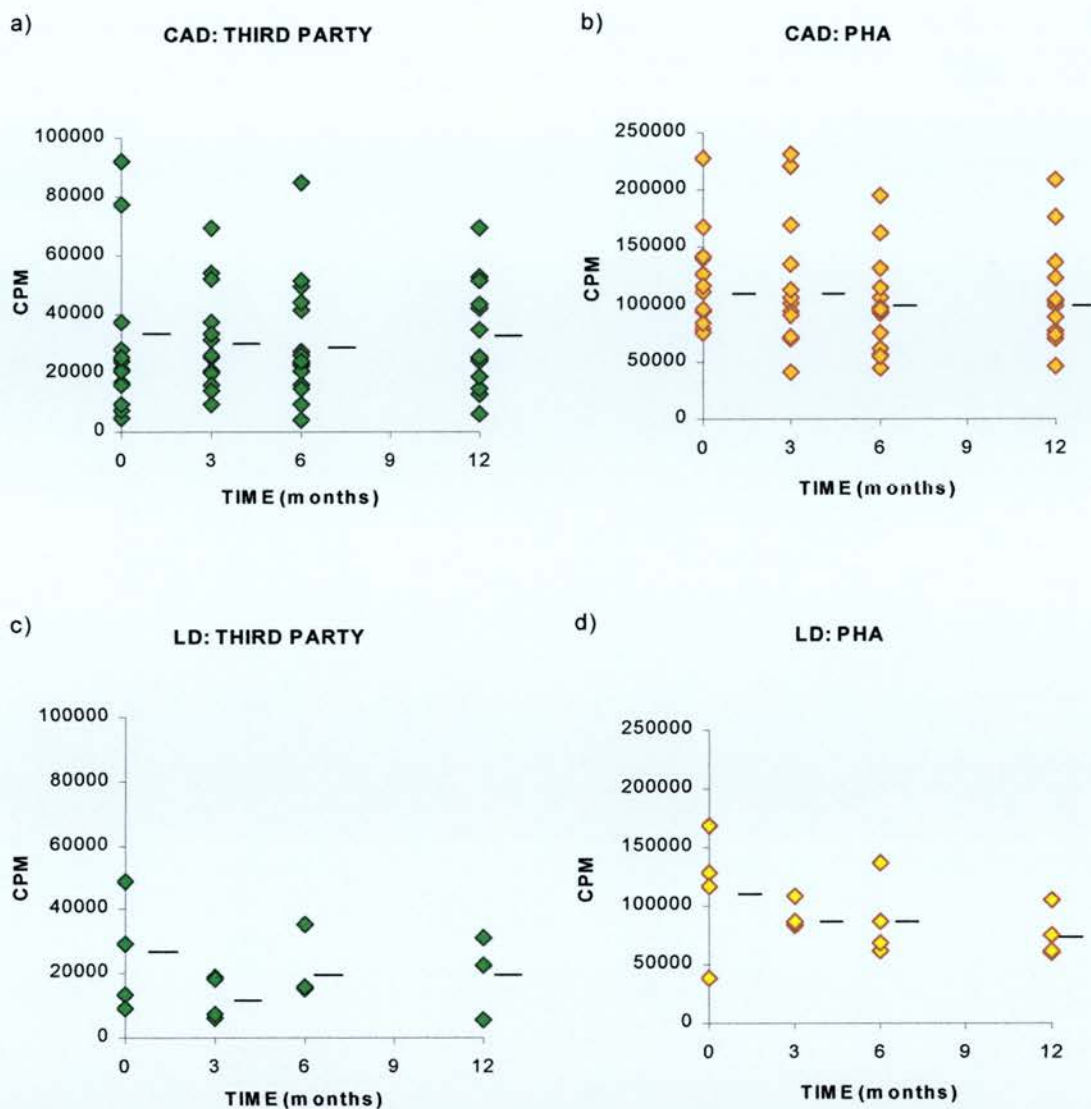


Figure 6.3: Proliferative responses of cadaveric (CAD) and living-donor (LD) recipients to stimulation with third party cells and PHA. Results shown are the mean ^3H -TdR incorporation of triplicate cultures of recipient responder cells after stimulation with third party cells (a and c) or PHA (b and d) in CAD (n=14) and LD (n=4) recipients respectively. Group means are represented by the bars.

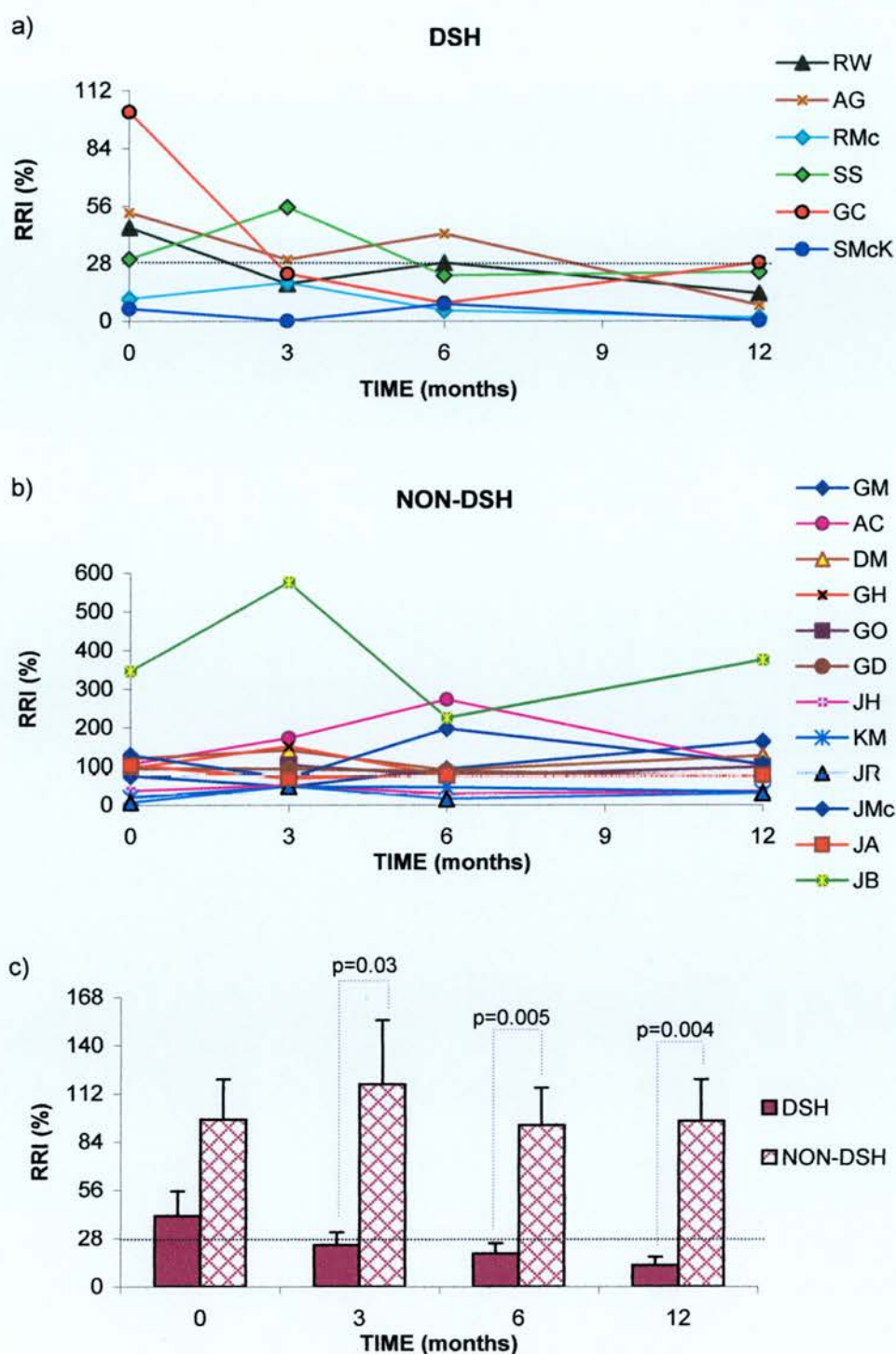


Figure 6.4: Relative response indices (RRI) of patients followed prospectively who developed DSH (n=6) compared with those who did not (n=12). Results shown are (a) the RRI of individual patients over the first 12 months post transplant of patients who developed DSH and (b) those who did not. (c) The mean RRI \pm SEM of patients in both groups throughout the time course, where DSH is represented by the dotted line.

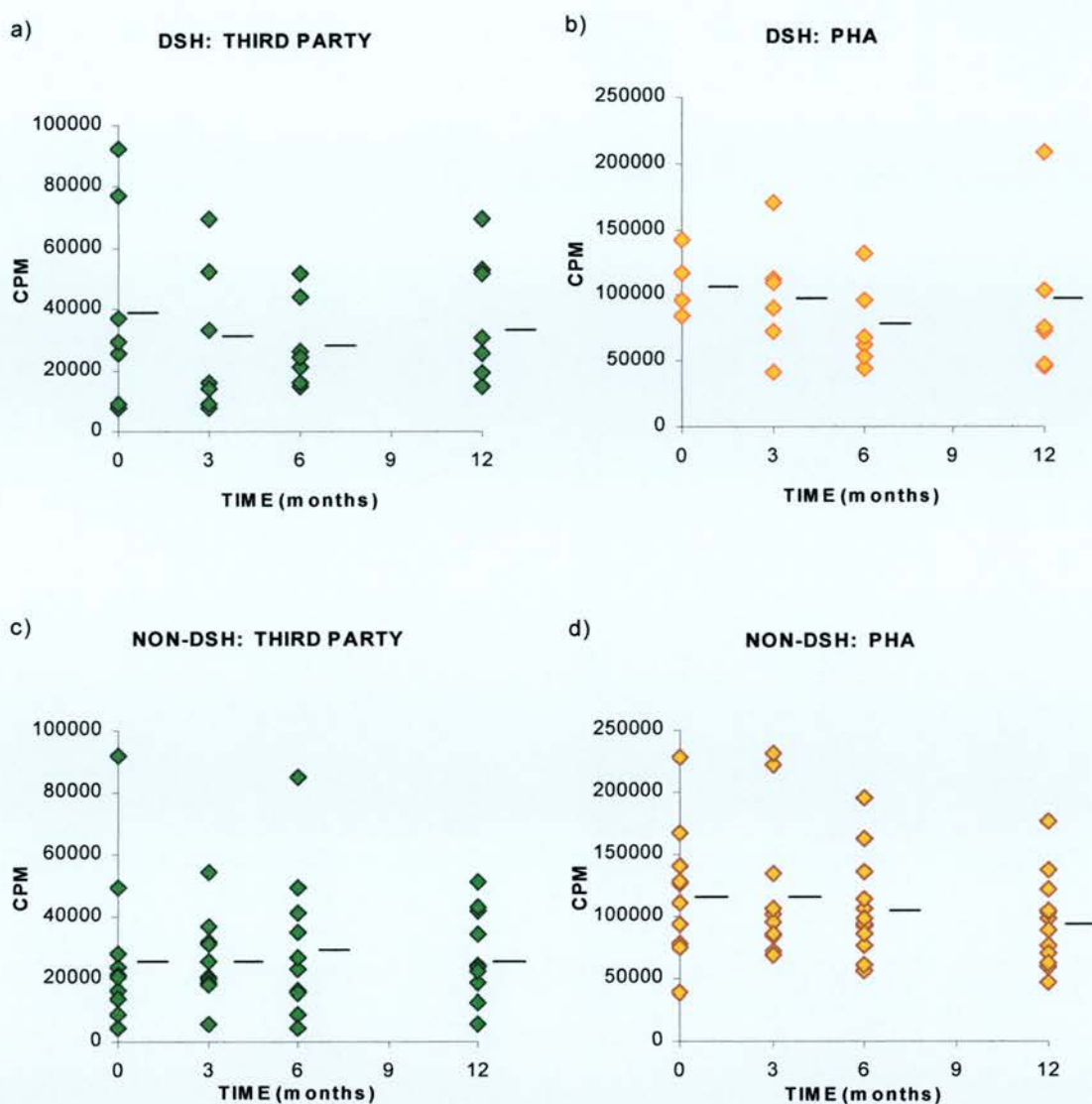


Figure 6.5: Proliferative response to third party stimulator cells and to PHA in patients who showed donor-specific hyporesponsiveness (DSH) compared with those who did not. Results shown are mean ^3H -TdR incorporation of triplicate cultures of recipient responder cells after stimulation with third party cells (a and c) or PHA (b and d) in patients who show DSH (n=6) and those who do not show DSH (n=12). Group means are represented by the bars.

PRODUCTION OF CYTOKINES

CADAVERIC GROUP

The predominant donor-specific cytokine produced pre-transplant in cadaveric recipients was IFN- γ , although this was only observed in 8/14 patients and acute rejection only occurred in two of these patients (Figure 6.6a). The level of IFN- γ secretion fell after 3 months, but there were no significant differences at any time point. Although IFN- γ also appeared to be the predominant cytokine at 12 months post-transplant, this was seen in only 7/14 patients. Pre-transplant, IFN- γ production after donor stimulation exceeded the levels produced after third party stimulation, but this difference was not significant and was only observed in 5/14 patients (Figure 6.6b). IFN- γ production did not appear to correlate with acute rejection, as only two of these patients experienced acute rejection. Similar levels of IFN- γ were produced at 3, 6 and 12 months post-transplant after donor and third party stimulation.

Donor-specific IL-2 production by cadaveric recipients remained high in the first 3 months and fell after 6 months post-transplant, although this change was not statistically significant (Figure 6.6a). IL-2 was the predominant cytokine in response to donor stimulation pre-transplant in three patients (GO, AG and GH), with levels exceeding those after third party stimulation, all of whom experienced early acute rejection. As a group, IL-2 production in response to third party was higher than after donor stimulation at all time-points within the first year post-transplant, but this was only statistically significant at 12 months post-transplant ($p=0.02$, Figure 6.6b). However, at 12 months post-transplant, the only patient (KM) who produced higher levels of IL-2 with donor stimulation compared with third party stimulation had stable graft function and no acute rejection episodes.

Although the level of donor-specific IL-4 production among cadaveric recipients appeared to rise at 12 months post-transplant, there were no significant differences in IL-4 production throughout the first year post-transplant (Figure 6.6c). IL-4 was the predominant cytokine in only one patient (AG) pre-transplant, who experienced no acute rejection, maintained stable graft function and developed DSH. In general, IL-4 production after donor stimulation exceeded the levels produced after third party stimulation at 6 and 12 months post-transplant, but these differences were not statistically significant and were seen in only 7/14 patients at each time point (Figure 6.6d).

Donor-specific IL-10 production appeared to fall after 3 months and then plateaued between 3 and 12 months post-transplant, but these differences did not reach statistical significance (Figure 6.6c). IL-10 was the predominant cytokine in only one patient (KM) pre-transplant, who experienced no acute rejection episodes. IL-10 production in response to third party stimulation exceeded that produced after donor stimulation in the first 3 months post-transplant, but this was seen in only 6/14 patients, and did not reach statistical significance (Figure 6.6d).

As I found in the cadaveric cohort of the Retrospective study, patients in the prospective cohort with a good graft outcome at 12 months post-transplant (Group A, n=9) also appeared to produce lower levels of donor-specific IL-2 and higher levels of IL-4 than patients with poor graft outcome (Group B, n=3) (Figure 6.7). However, these differences were not statistically significant as the sample size in Group B was small.

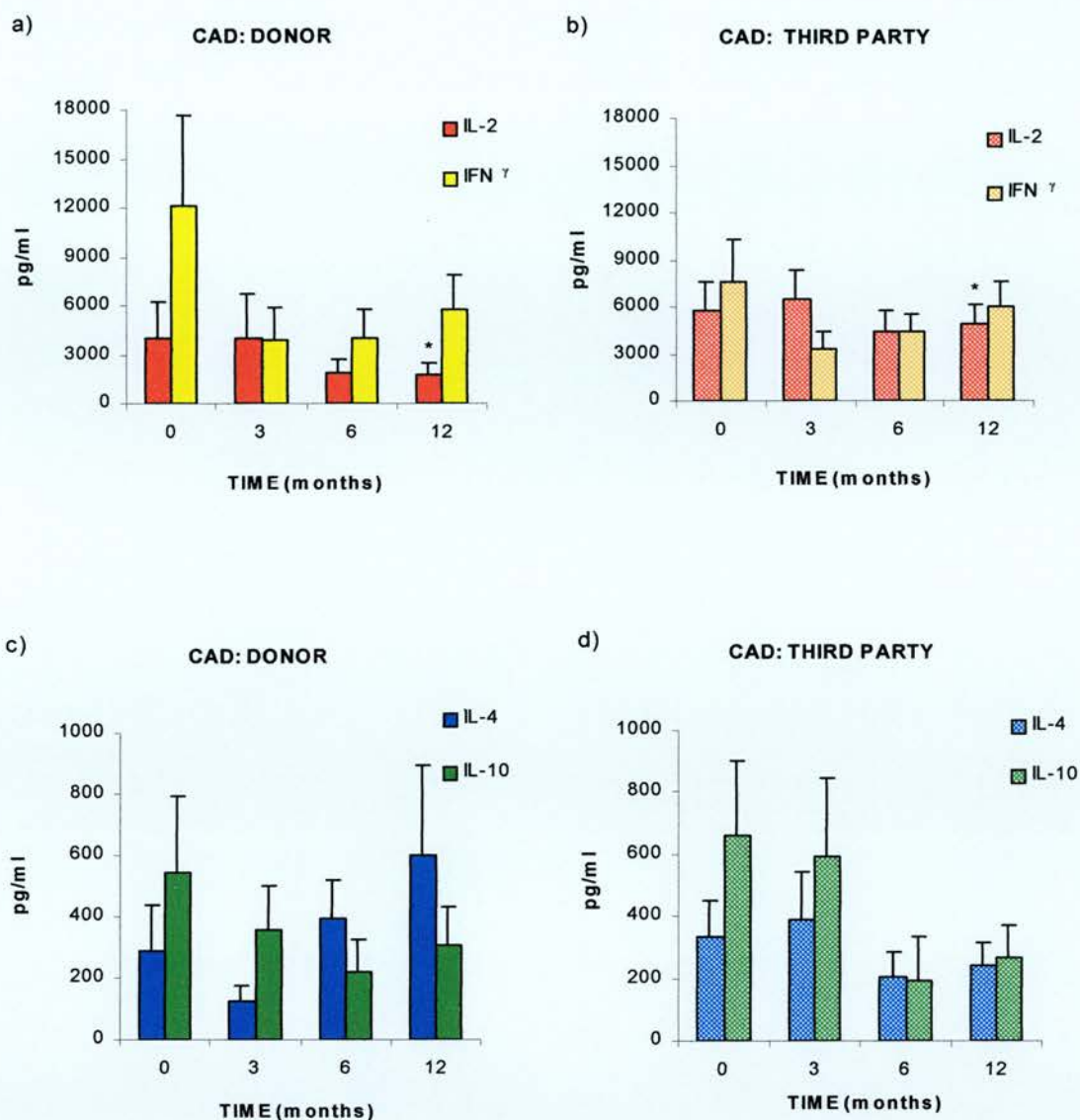


Figure 6.6: Cytokine production by cadaveric (CAD) recipients (n=14) during the first 12 months post-transplant. Results shown are mean cytokine production after stimulation with donor (a and c) and third party (b and d) cells \pm SEM after subtraction of the background responses in the presence of autologous stimulators. (*p<0.05)

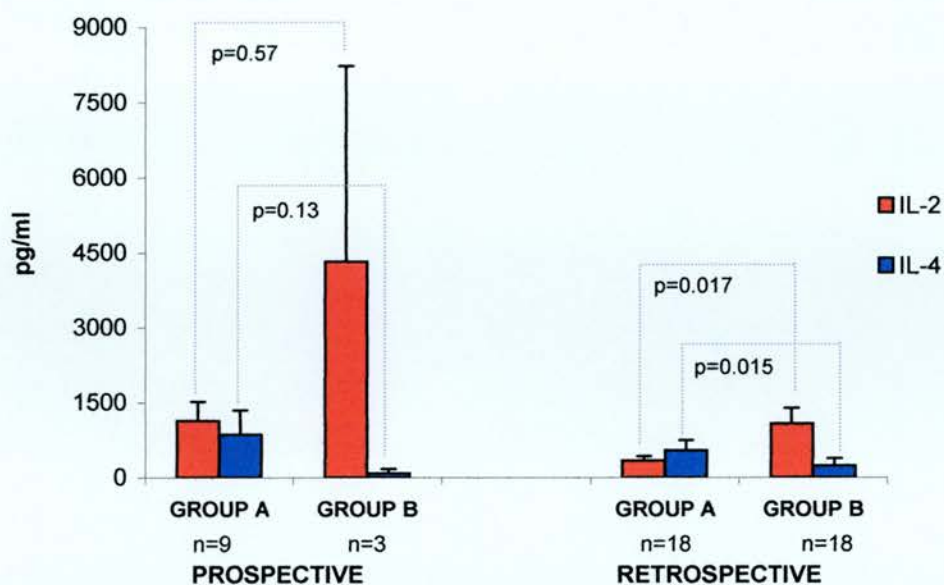


Figure 6.7: Donor-specific cytokine production in cadaveric recipients at 12 months post-transplant in the prospective cohort compared with the retrospective cohort based on graft outcome. Results shown are mean donor-specific IL-2 and IL-4 production \pm SEM after subtraction of the background responses in the presence of autologous stimulators in patients with good graft outcome (Group A) compared with patients with poor graft outcome (Group B).

LIVING-DONOR GROUP

Low levels of all cytokines were produced pre-transplant in LD recipients in response to donor stimulation. Levels of IL-2 remained low throughout the first 12 months post-transplant (Figure 6.8a), especially when compared with their cadaveric counterparts. Surprisingly, donor-specific IL-2 production exceeded that produced after third party stimulation at all time-points, but these differences were not statistically significant (Figure 6.8b). As in the cadaveric recipients, donor-specific IL-2 production pre-transplantation appeared to correlate with acute rejection episodes. Indeed, 3/4 LD recipients (JA, JMCL and JB) showed higher IL-2 production after donor stimulation compared with third party stimulation before transplantation and all of these patients experienced acute rejection. This trend was maintained throughout the first 12 months post-transplant in two patients (JMCL and JB).

Donor-specific IFN- γ levels were also low, but increased somewhat during the first year post-transplant (Figure 6.8a). Although IFN- γ production before transplantation was higher in response to third party than after donor stimulation, by 12 months post-transplant, donor-specific IFN- γ production exceeded that found after third party stimulation, but these differences were not statistically significant (Figure 6.6b). Similar to IL-2 production, two patients (JMCL and JB) produced higher levels of IFN- γ after donor compared with third party stimulation at 6 and 12 months post-transplant.

No IL-4 was produced in response to donor stimulation before transplantation in any of the patients, but the level increased throughout the first 12 months post-transplant (Figure 6.8c). At 12 months post-transplant, IL-4 production after donor

stimulation exceeded that produced after third party stimulation, but this was not statistically significant (Figure 6.8d). Donor-specific IL-10 production appeared to increase at 3 months post-transplant, but levels returned to baseline by 12 months post-transplant (Figure 6.8c). IL-10 production in response to donor cells exceeded third party stimulation at all time-points, but these differences were not statistically significant (Figure 6.8d). Because of the small numbers of patients, I was unable to perform a sub-group analysis based on graft outcome in the living-donor recipients in the prospective study.

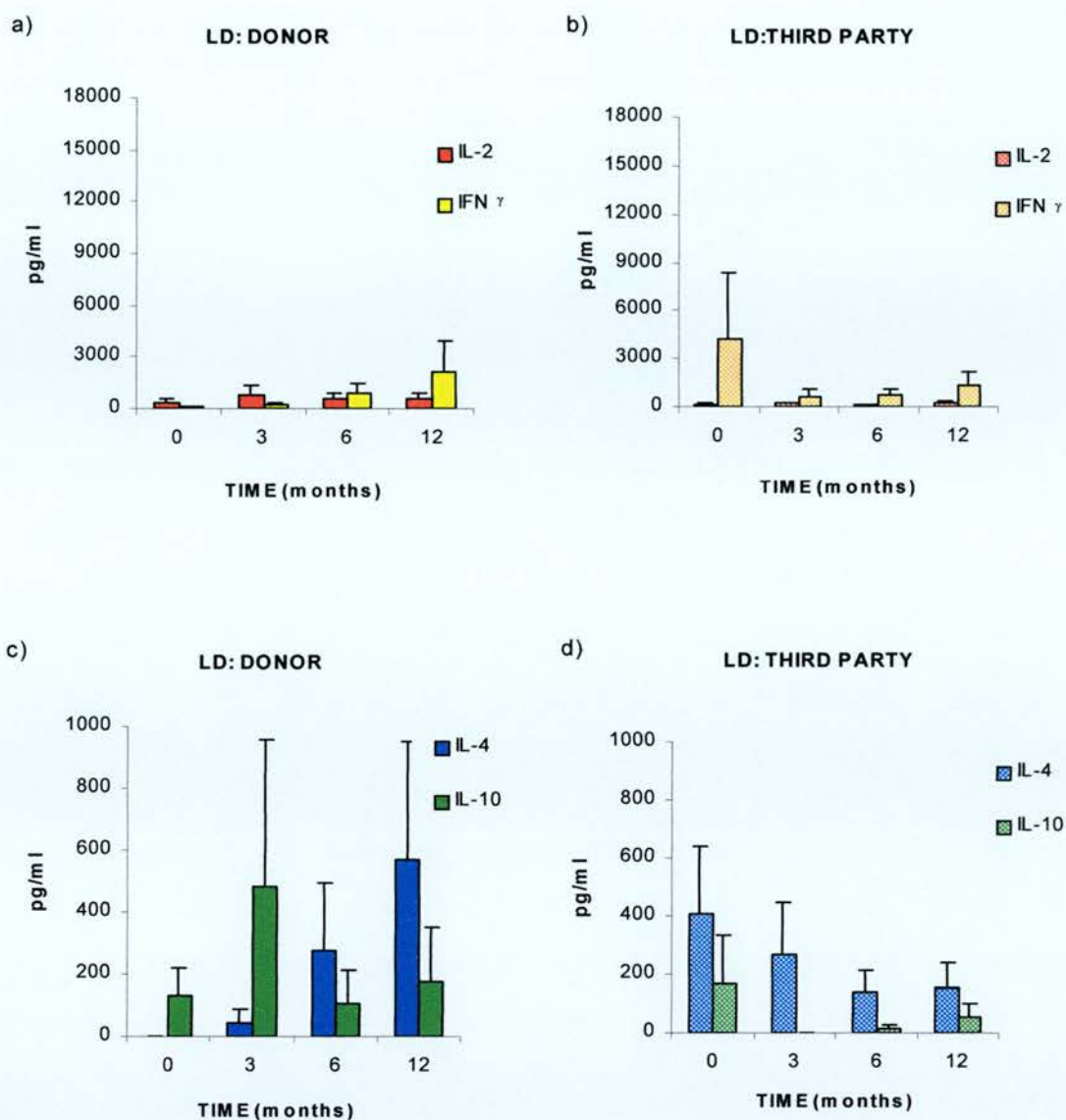


Figure 6.8: Cytokine production by living-donor (LD) recipients (n=4) during the first 12 months post-transplant. Results shown are mean cytokine production after stimulation with donor (a and c) and third party (b and d) cells \pm SEM after subtraction of the background responses in the presence of autologous stimulators.

GRAFT LOSS RECIPIENTS

Three patients had early graft loss as a consequence of acute rejection, but donor cells were available for only two patients. The first patient NP, aged 34 years, received a cadaveric graft which was poorly HLA-matched, had delayed graft function and developed severe acute rejection with infarction necessitating graft nephrectomy. The second patient CH, aged 35 years, received an HLA-haploidentical graft from his brother, had immediate graft function but lost the graft as a result of steroid-resistant acute rejection.

The proliferative responses pre-transplant by both patients to stimulation with their respective donors exceeded third party stimulation, yielding an RRI of 146% for NP and 111% for CH. In comparison to the cadaveric recipients who maintained their grafts, only 3/14 of these patients showed an RRI exceeding 100%. NP and CH showed good proliferative responses after stimulation with third party cells and PHA. Cytokine analysis was performed on NP only, as recipient cell numbers for CH were inadequate. Lymphocytes obtained pre-transplant from NP produced high levels of IL-2 and IFN- γ after stimulation with donor cells (Figure 6.9a), and these levels were over two-fold higher than the mean cytokine production by cadaveric recipients who maintained their grafts. Donor-specific secretion of IL-2 and IFN- γ also exceeded that produced following third party stimulation (Figure 6.9b), but was lower than that produced after stimulation with PHA (Figure 6.9c). NP produced very low levels of IL-4 and IL-10 in response to donor stimulation pre-transplant.

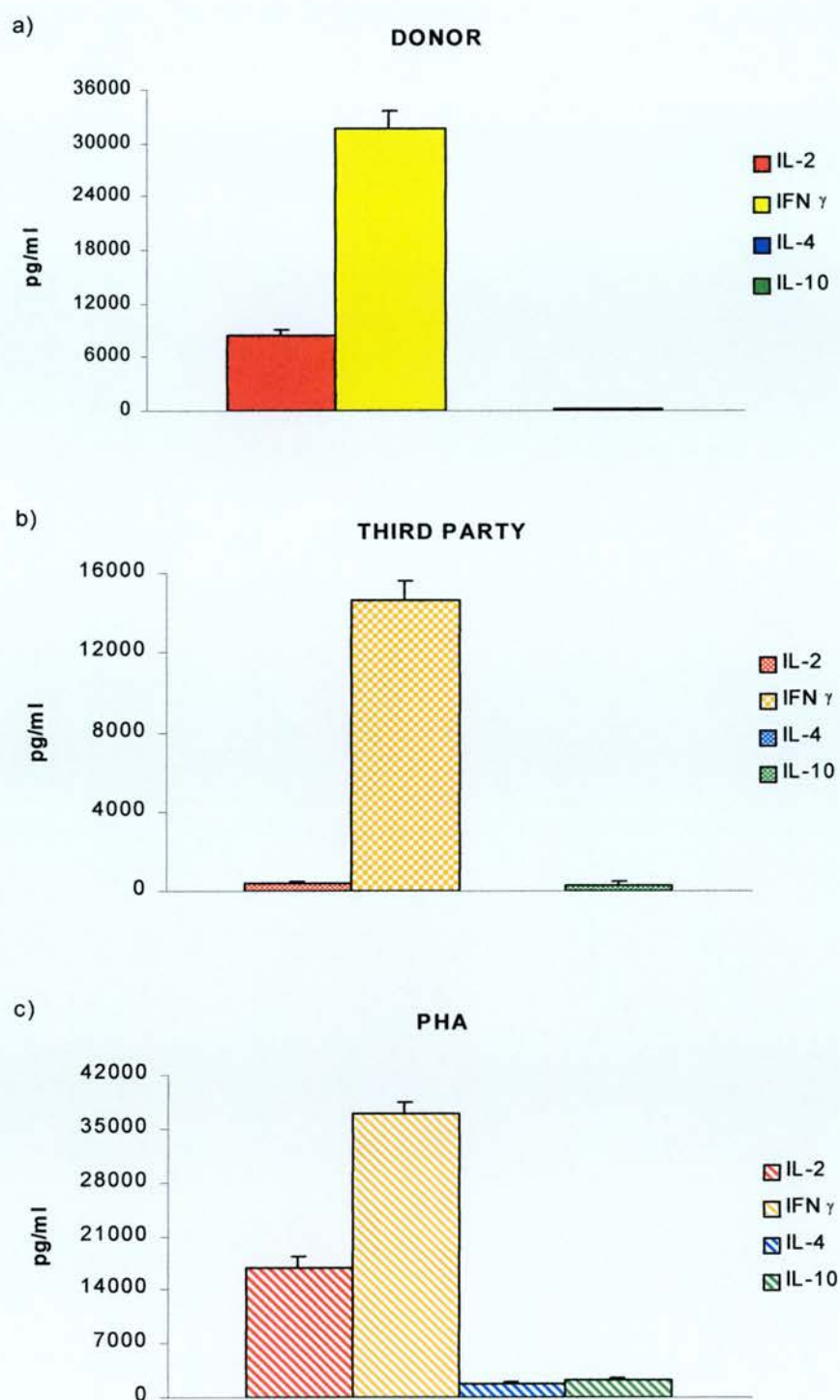


Figure 6.9: Cytokine production in a cadaveric recipient, NP, with early graft loss secondary to severe acute rejection. Results shown are mean cytokine concentrations of triplicate cultures in response to stimulation with donor cells (a), third party cells (b) and PHA (c) immediately pre-transplant.

EXPRESSION OF CYTOKINE mRNA

As RT-PCR analysis had proved rather unreliable as a method for assessing cytokine production in the previous arm of the study, I selected only two patients in the prospective arm for PCR analysis. One of these, RW showed DSH (Figure 6.10a) and the other, AC, did not (Figure 6.10b). IL-4 and TGF- β mRNA expression were not assessed due to the problems I encountered with these in the retrospective cohort. IL-4 proved difficult to detect despite many attempts to standardise the assay and TGF- β mRNA expression was difficult to differentiate as it was found in cells stimulated with autologous cells as well as the MLR.

IL-2

In the patient who showed DSH, donor-specific IL-2 mRNA expression was only detected in the pre-transplant sample (RW, Figure 6.10a). This was consistent with the results obtained by ELISA. IL-2 mRNA was detected in third party stimulated samples, except at 3 months post-transplant, and in all PHA stimulated samples. In the patient who did not show DSH, donor-specific IL-2 mRNA expression and high levels of donor-specific IL-2 production were detected pre-transplant and at all time points post-transplant (AC, Figure 6.10b). IL-2 mRNA was detected in all third party stimulated samples, again apart from at 3 months post-transplant, and in all PHA stimulated samples.

IFN- γ

In the patient who showed DSH, donor-specific IFN- γ mRNA expression and IFN- γ production were detected pre-transplant and at all time points post-transplant (RW,

Figure 6.10a). IFN- γ mRNA was also detected in all third party and PHA stimulated samples. In the patient who did not show DSH, donor-specific IFN- γ mRNA was also detected at all time points and high levels of IFN- γ production were found throughout the first year post-transplant (AC, Figure 6.10b). IFN- γ mRNA was only detected in third party stimulated samples pre-transplant and at 12 months post-transplant, but was present in all PHA stimulated samples.

IL-10

The patient who developed DSH showed IL-10 mRNA expression pre-transplant and at 3 months post-transplant (RW, Figure 6.10a). IL-10 production was detectable by ELISA pre-transplant and at 12 months post-transplant only. IL-10 mRNA was detected in all third party and PHA stimulated samples. The patient who did not develop DSH showed IL-10 mRNA expression at all time points, with high levels of IL-10 measured by ELISA pre-transplant and also at 3 and 6 months post-transplant (AC, Figure 6.10b), but no IL-10 was produced at 12 months post-transplant. IL-10 mRNA was detected in all third party stimulated samples, apart from 3 months post-transplant, and in all PHA stimulated cells.

These results showed generally good correlation between donor-specific cytokine gene expression and cytokine production as measured by ELISA in patients followed prospectively. However, discordant results were obtained for some third party stimulated cells for both patients. Therefore, like the retrospective study, PCR studies were not pursued.

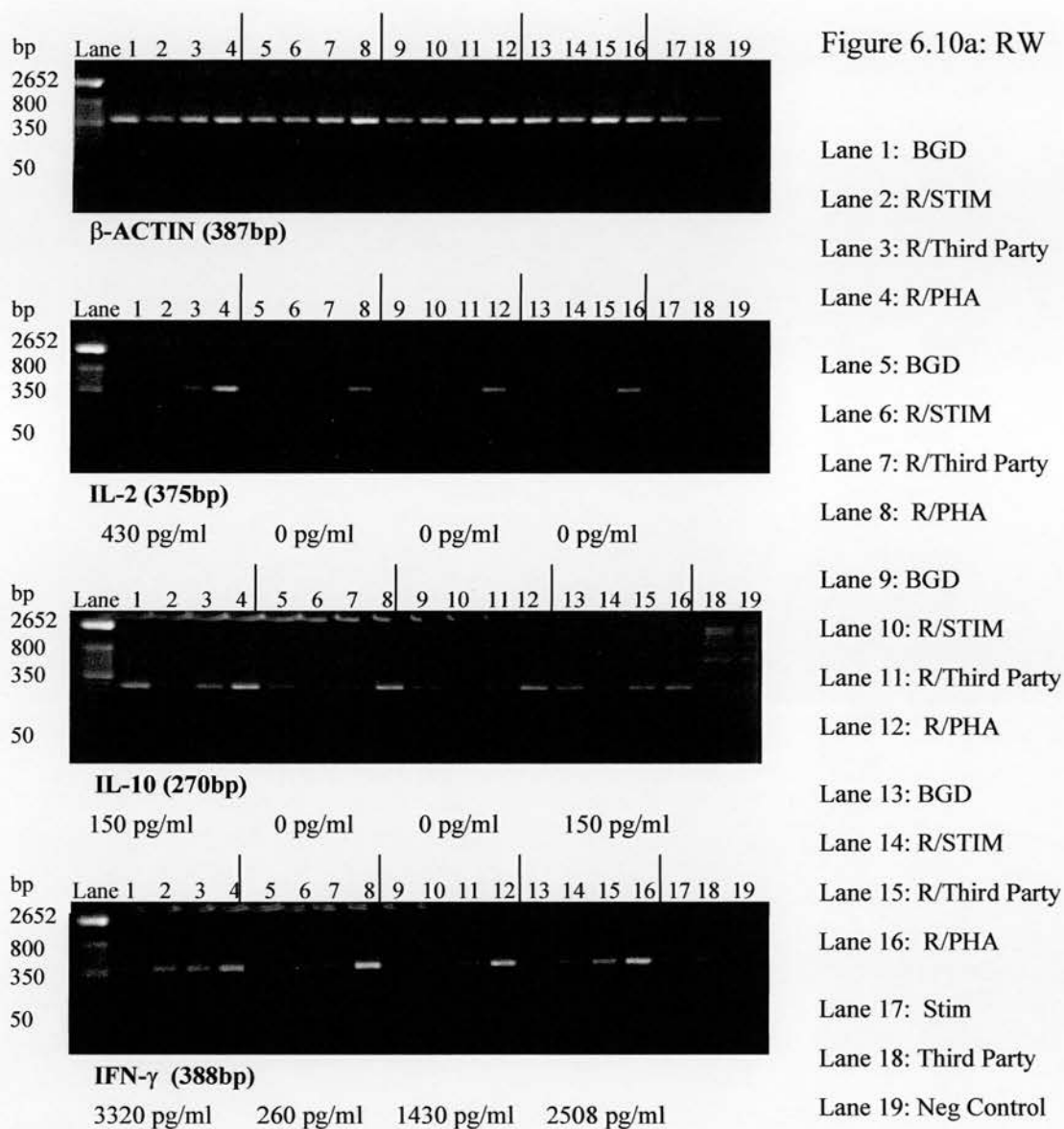


Figure 6.10a: IL-2, IL-10, and IFN- γ mRNA expression measured by RT-PCR in cells from a cadaveric recipient followed prospectively for 12 months who showed DSH. Lanes 1-4: pre-transplant, Lanes 5-8: 3 months post-transplant, Lanes 9-12: 6 months post-transplant and Lanes 13-16: 12 months post-transplant. BGD - autologous control, R/STIM - donor-stimulated cells, R/Pool - third party stimulated cells, R/PHA - PHA stimulated cells. Lane 17 - stimulator cells (limited RNA available), Lane 18 - Third party cells (Pool), Lane 19 - negative control.

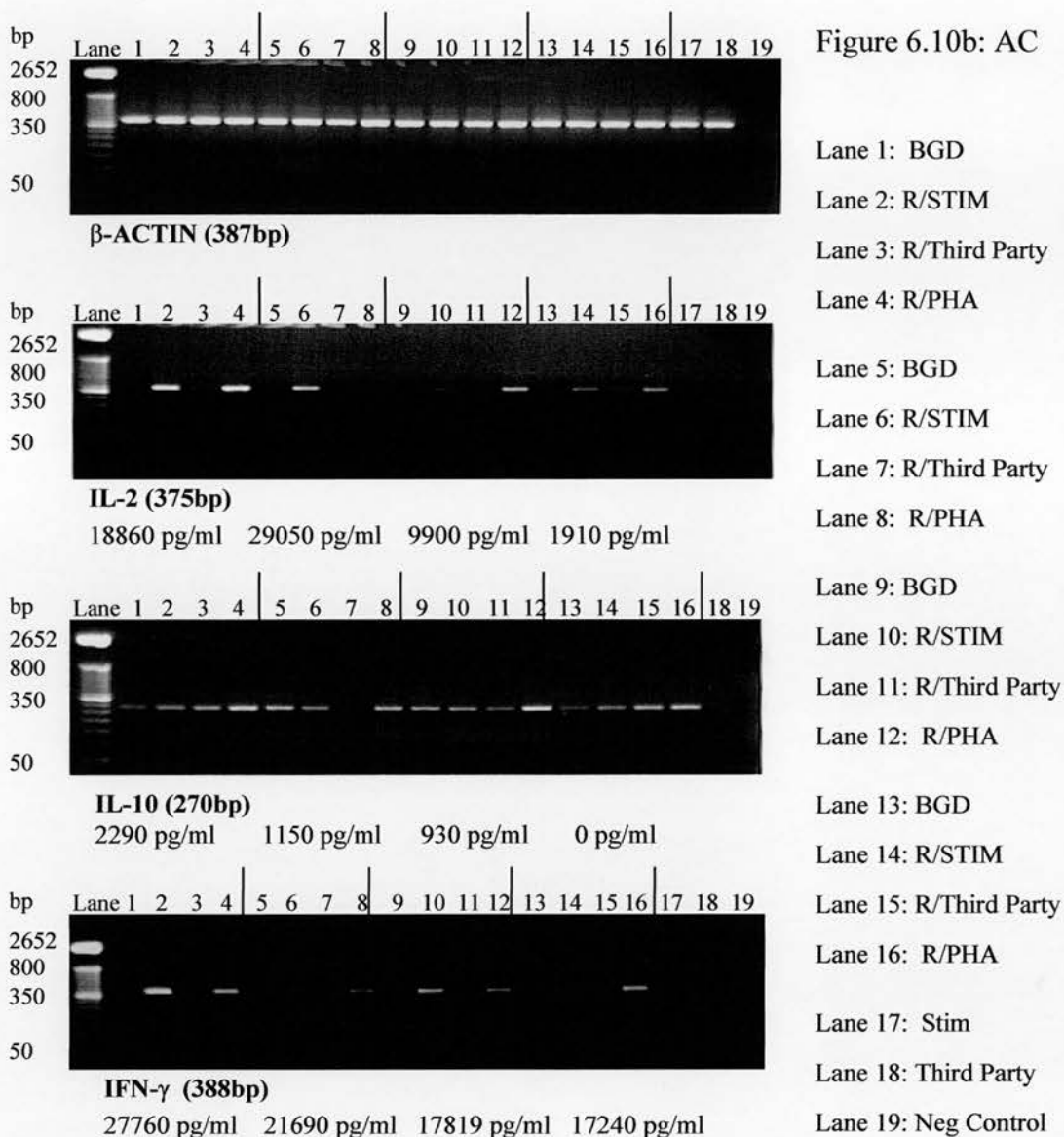


Figure 6.10b: IL-2, IL-10, and IFN- γ mRNA expression measured by RT-PCR in cells from a cadaveric recipient followed prospectively for 12 months who did not show DSH. Lanes 1-4: pre-transplant, Lanes 5-8: 3 months post-transplant, Lanes 9-12: 6 months post-transplant and Lanes 13-16: 12 months post-transplant. BGD - autologous control, R/STIM - donor-stimulated cells, R/Pool - third party stimulated cells, R/PHA - PHA stimulated cells. Lane 17 - stimulator cells (limited RNA available), Lane 18 - Third party cells (Pool), Lane 19 - negative control.

CLINICAL FEATURES OF DSH

As in the retrospective study, I compared the clinical and immunological features of patients with and without DSH. When patients were divided according to the presence of or absence of DSH (defined by an RRI $\leq 28\%$) irrespective of graft function, no significant differences were found with respect to age, degree of HLA-mismatch, level of sensitisation as measured by PRA, or serum creatinine at one year post-transplant (Table 6.4). Only 3/6 patients who showed DSH maintained good graft function (serum creatinine $< 140 \mu\text{mol/L}$) at 12 months post-transplant. As noted above, I monitored graft function for a further year to assess graft outcome beyond the period of in vitro analysis, but there was no significant difference in the level of serum creatinine between patients with DSH and those without DSH throughout the first two years post-transplant (Figure 6.11).

The immunosuppressive therapy load was comparable between patients with DSH and those without DSH, all patients being maintained on standard triple therapy (prednisolone, azathioprine and cyclosporin). However, the incidence of acute rejection was significantly lower among patients who developed DSH than those who did not (17% versus 58% respectively, $p=0.01$, X^2 test). One patient from each group developed biopsy-proven chronic rejection at the end of the 12-month follow up period.

Table 6.4: Clinical features of patients who developed DSH within the first 12 months post-transplant and those who did not.

GROUP	AGE (years)	M/M	PRA (%)	AR		CR	12mth - CREAT (μ mol/L)
				EARLY	LATE		
DSH (n=6)	54.3 (18.4 - 64)	2 (1 - 5) 1 ID	1 (0 - 32)	1	0	1	141 (95 - 176)
NON-DSH (n=12)	35.4 (24.6 - 60.3)	2 (0 - 6) 2 HI, 1 NI	2 (0 - 66)	5	2	1	129 (102 - 280)
p value	0.45	0.96	0.94				0.73

AGE TX: age at transplant, M/M: degree of HLA-A, B and DR-mismatch, PRA: panel reactive antibody level, AR: acute rejection, CR: chronic rejection, 12mth-CREAT: creatinine at 12 months.

DSH: donor-specific hyporesponsiveness, NON-DSH: donor-specific hyporesponsiveness not found.

*Results are shown in medians and ranges.

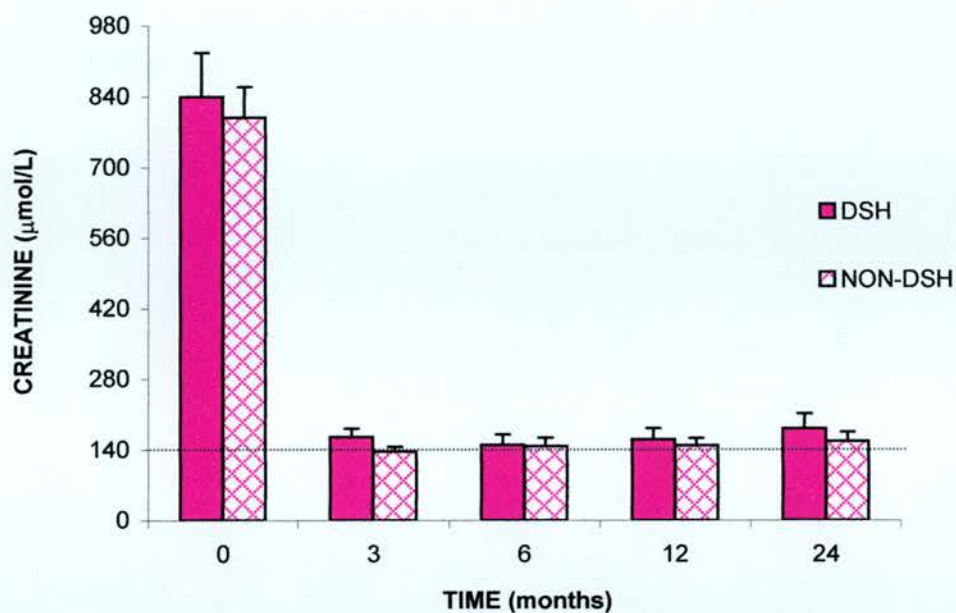


Figure 6.11: Graft function during the first 2 years post-transplant of patients followed prospectively who showed DSH (n=6) compared with those who did not (n=12). Results shown are the mean serum creatinine \pm SEM over the first 24 months post-transplant. The dotted line represents good graft function (<140 mmol/L).

IMMUNOLOGICAL FEATURES OF DSH

Next, I compared the cytokine profiles of patients who showed DSH with those who did not. Patients who showed DSH produced low donor-specific levels of IL-2 pre-transplant and throughout the 12-month follow up period (Figure 6.12a). All these patients produced higher levels of IL-2 in response to third party stimulation than donor stimulation at all time points (Figure 6.12b). Pre-transplant IFN- γ production in this group was higher in response to third party compared with donor stimulation in all patients who developed DSH (Figures 6.12a and 6.12b). Thereafter, both donor and third party IFN- γ production fell, although these differences were not statistically significant. Donor-specific IL-4 levels in patients with DSH appeared to increase post-transplant and at 12 months post-transplant exceeded third party production, but these differences did not reach statistical significance (Figures 6.12c and 6.12d). At the end of the study period, donor-specific IL-10 production remained essentially unchanged compared with pre-transplant levels and was lower than third party production in patients who showed DSH (Figure 6.12c).

Patients who did not develop DSH within the first year post-transplant produced high levels of IL-2 in response to donor stimulation before transplant, although IL-2 production then decreased during the study period (Figure 6.13a). Similar to the DSH group, third party-specific IL-2 production was higher than donor-specific IL-2 production at all time points in patients who did not develop DSH (Figure 6.13b). As in the patients who developed DSH, donor-specific IFN- γ production was highest immediately pre-transplant and decreased after 3 months post-transplant (Figure 6.13a). However, unlike the DSH group, patients who did not develop DSH produced higher levels of IFN- γ pre-transplant after donor stimulation

than after third party stimulation, but this was not statistically significant (Figure 6.13b). As in the patients with DSH, donor-specific IL-4 production appeared to increase throughout the first year post-transplant and exceeded third party production at 12 months post-transplant, but these differences were not statistically significant (Figures 6.13c and 6.13d). IL-10 production in response to donor and third party decreased throughout the study period, but again these differences were not statistically significant (Figures 6.13c and 6.13d).

When donor-specific cytokine production by patients who showed DSH and those who did not were compared directly, IL-2 production was lower in patients who showed DSH compared with those who did not at all time points (Figure 6.14a). However this was statistically significant only in pre-transplant samples. IFN- γ production was also lower in patients who showed DSH throughout the study period, reaching statistical significance at 6 and 12 months post-transplant (Figure 6.14b). IL-4 production pre-transplant and at 12 months post-transplant appeared to be higher in patients who showed DSH than those who did not, but this was not statistically significant (Figure 6.14c). Patients who developed DSH produced lower levels of IL-10 pre-transplant than patients who did not develop DSH, although similar levels of IL-10 were produced by the two groups at 12 months post-transplant (Figure 6.14d).

In the retrospective arm of the study, I found that long-term cadaveric graft survivors who showed DSH produced two-fold higher levels of donor-specific IL-4 than IL-2 and this was not seen in the patients who did not show DSH. In the prospective study, patients who developed DSH produced 2.4 times higher levels of IL-4 than IL-2 immediately pre-transplant, but only 1.3 times higher levels of IL-4

than IL-2 at 12 months post-transplant as a group (Figure 6.15). As in the retrospective study, good graft outcome was also associated with a trend towards low IL-2 and high IL-4 production in the prospective cohort.

Only three of the patients in the prospective study developed DSH and maintained good graft function at 12 months post-transplant. All were recipients of cadaveric grafts. These patients showed a predominance of donor-specific IL-4 production over IL-2 production at 12 months post-transplant (Figure 6.16). At the end of the study period, patient RW produced IL-4 but no IL-2, patient GC produced 4.2 times more IL-4 than IL-2 and patient AG produced 3.1 times more IL-4 than IL-2. The three remaining patients with poor graft function who showed DSH produced higher levels of IL-2 than IL-4 at 12 months post-transplant.

Similar to the retrospective cadaveric cohort, I assessed the ratio of donor-specific IL-4/ IL-2 production in patients who showed DSH and those who did not depending on graft outcome. Patients who showed DSH and had a good graft outcome at 12 months post-transplant produced 4-fold higher levels of donor-specific IL-4 than IL-2 (Table 6.5). However, unlike the retrospective cohort, patients with DSH and poor graft outcome produced a similar IL-4/IL-2 ratio as patients who did not show DSH (Table 6.5). Therefore, unlike the retrospective cadaveric cohort, low IL-2 and high IL-4 production was seen only in DSH patients with good graft function.

Table 6.5: Ratio of donor-specific IL-4/ IL-2 production at 12 months post-transplant in patients who showed DSH and those who did not.

DSH			NON-DSH		
ALL n=6	GOOD F’N n=3	POOR F’N n=3	ALL n=12	GOOD F’N n=8	POOR F’N n=4
1.29	4.03	0.18	0.24	0.38	0.11

DSH: donor-specific hyporesponsiveness.

NON-DSH: donor-specific hyporesponsiveness not detected.

ALL: all patients in each cohort.

GOOD F’N: good graft function.

POOR F’N: poor graft function.

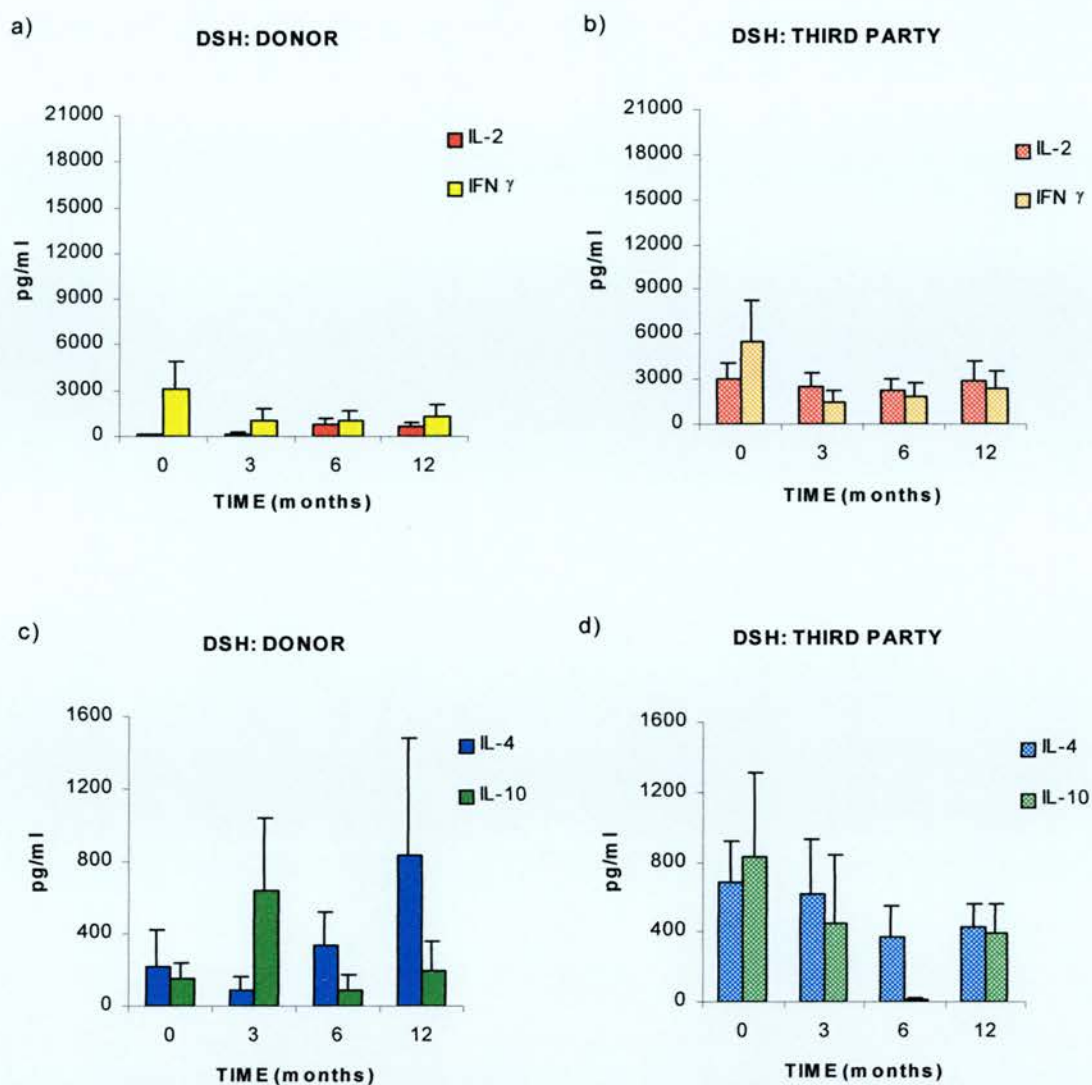


Figure 6.12: Cytokine production by patients who developed DSH (n=6) at 12 months post-transplant. Results shown are the mean cytokine production \pm SEM after subtraction of background responses in the presence of autologous stimulators after stimulation with donor cells (a and c) and third party cells (b and d).

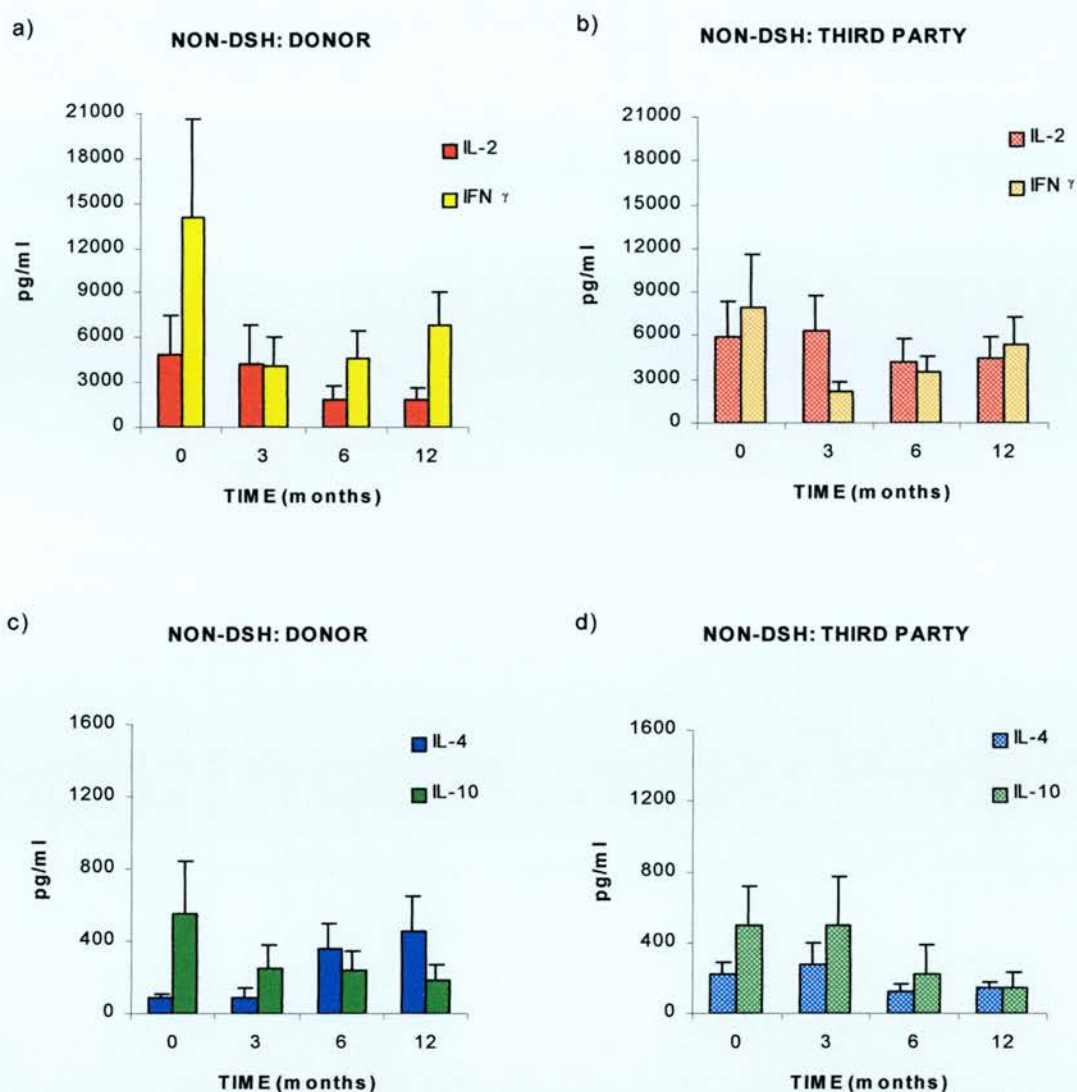


Figure 6.13: Cytokine production by patients who do not develop DSH (n=12) within the first year post-transplant. Results shown are mean cytokine production \pm SEM after subtraction of the background responses in the presence of autologous stimulators after stimulation with donor cells (a and c) and third party cells (b and d).

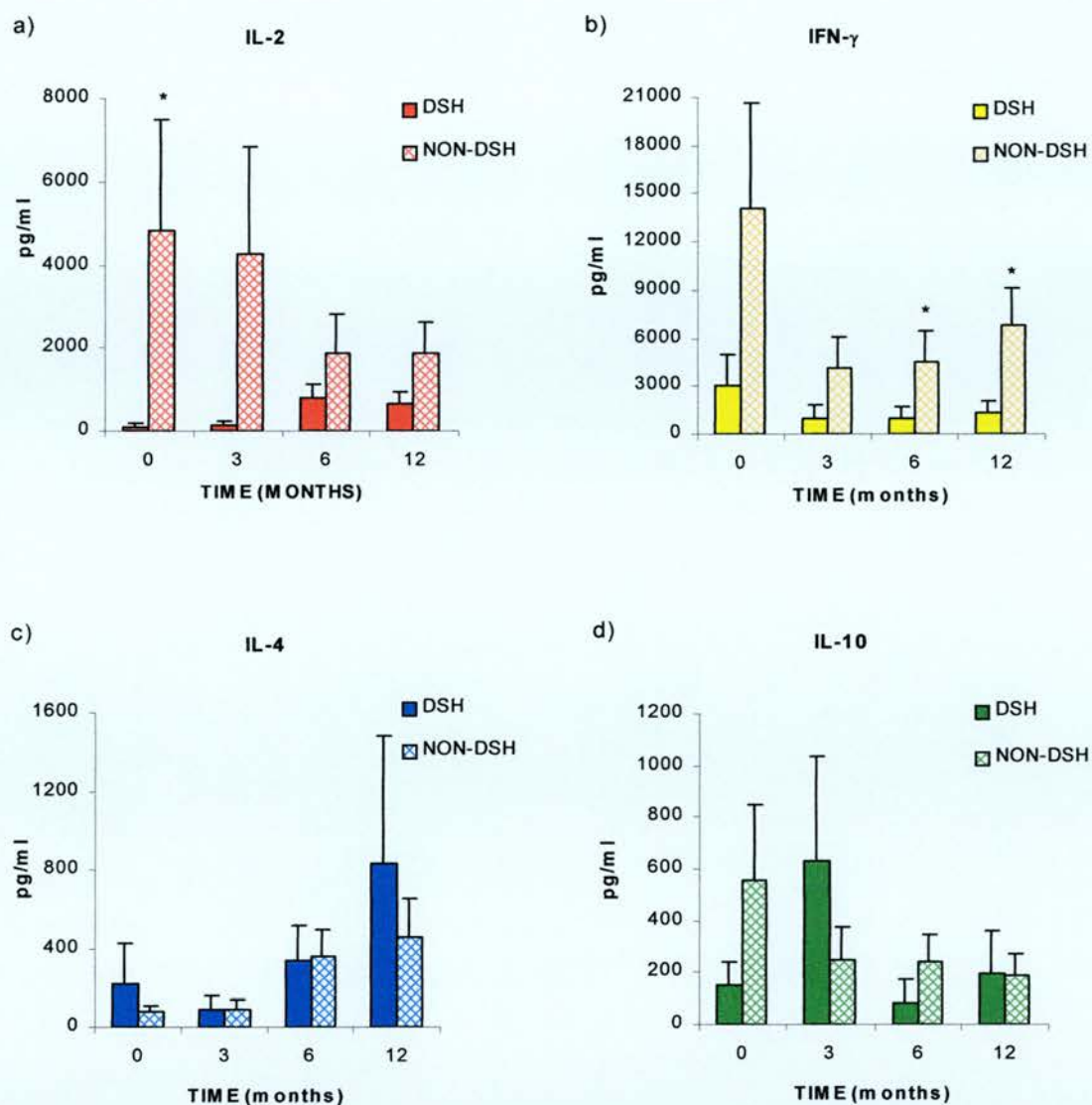


Figure 6.14: Donor-specific cytokine production during the first 12 months post-transplant of patients who developed DSH (n=6) compared with those who did not (n=12). Results shown are mean production of IL-2 (a), IFN- γ (b), IL-4 (c) and IL-10 (d) \pm SEM after subtraction of the background responses in the presence of autologous stimulators. (* $p < 0.05$)

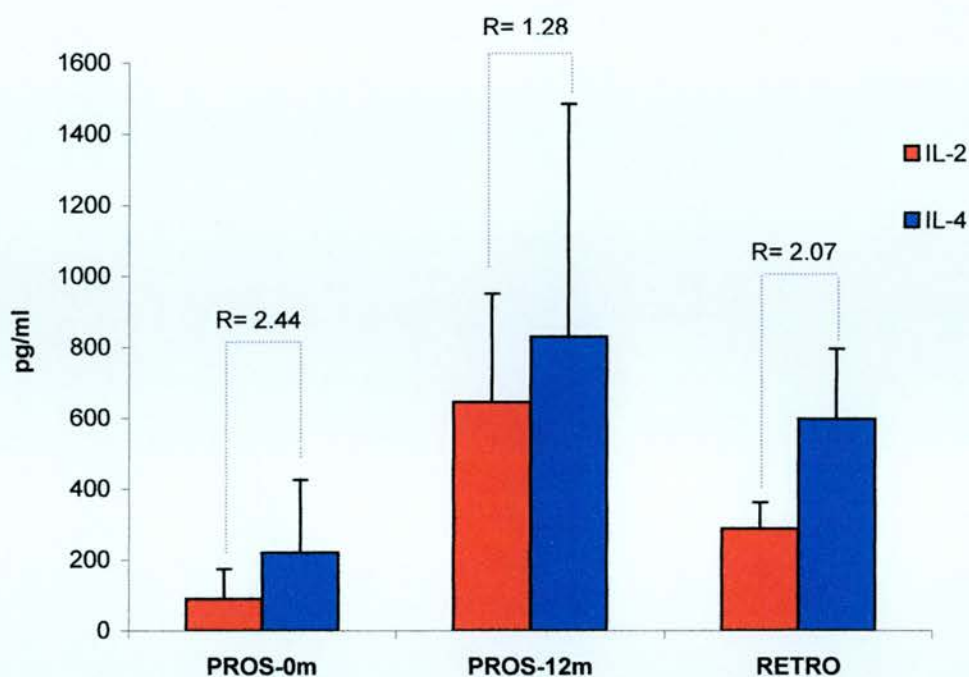


Figure 6.15: Donor-specific cytokine production of patients who show DSH in the prospective study (n=6) compared with those in the retrospective arm of the study (n=22). Results shown are the mean IL-2 and IL-4 production \pm SEM after subtraction of the background responses in the presence of autologous stimulators pre-transplant (PROS-0m) and at 12 months post-transplant (PROS-12m) in the prospective study, and in long-term graft survivors who show DSH in the retrospective study (RETRO). The ratio (R) of IL-4: IL-2 production is shown for each group.

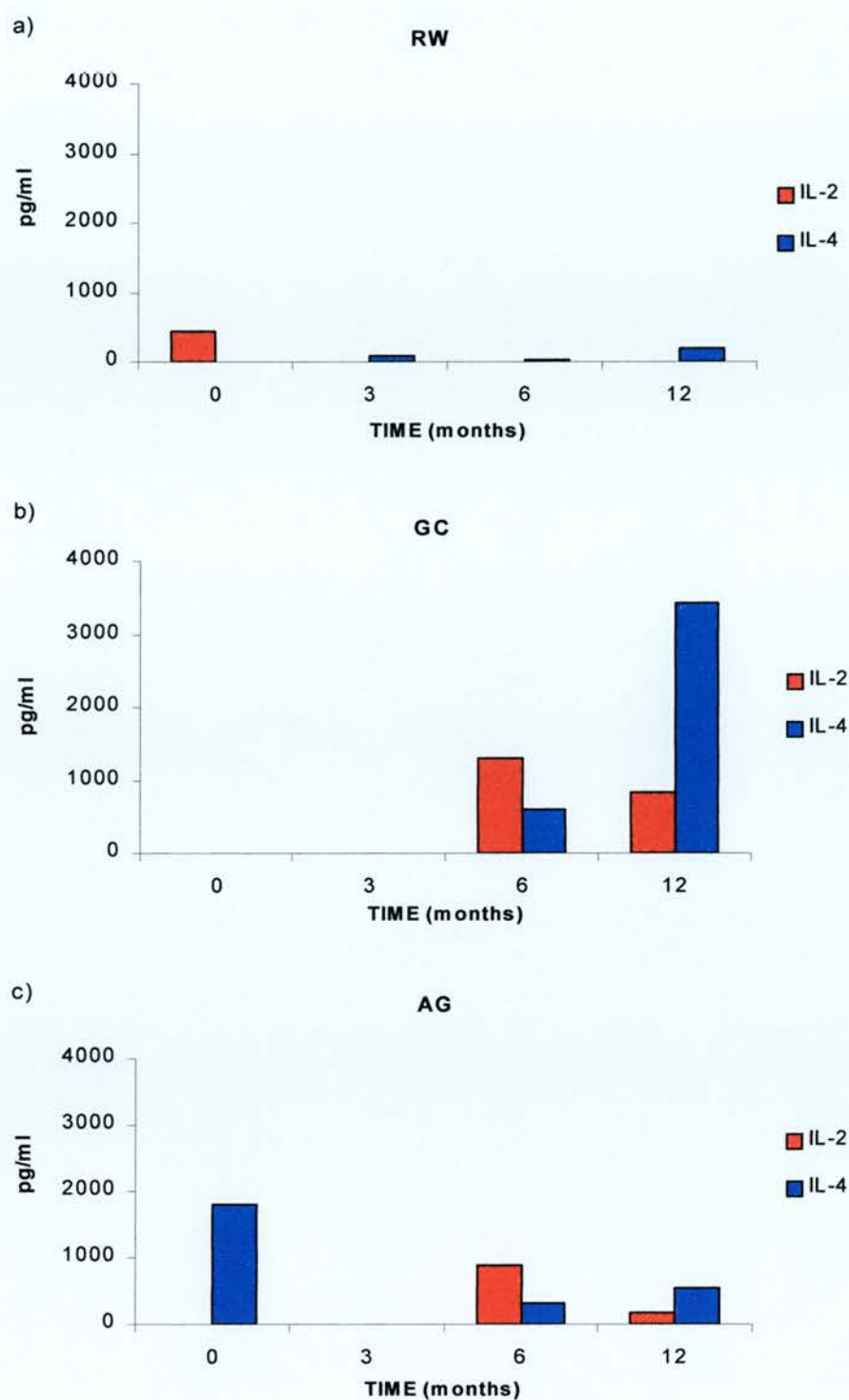


Figure 6.16: Donor-specific cytokine production of patients with good graft function who show DSH in the first 12 months post-transplant. Results shown are the mean IL-2 and IL-4 production throughout the first year post-transplant of the three patients with good graft function who showed DSH.

SUMMARY

The prospective arm of this study included 14 cadaveric and 4 living-donor recipients who completed one year of follow up. Although MLR-defined DSH was found in 6/18 of the patients, only half of these maintained good graft outcome at the end of the study period. As in the retrospective cadaveric cohort, good graft outcome in patients with DSH appeared to be associated with low IL-2 and high IL-4 production. Indeed, a predominance of donor-specific IL-2 production over third party production pre-transplantation appeared to correlate with acute rejection in both cadaveric and living-donor recipients.

All patients with DSH who maintained good graft function at one year post-transplant produced at least three-fold higher levels of donor-specific IL-4 than IL-2. Patients who developed DSH also produced lower levels of IFN- γ throughout the study period compared with those who did not develop DSH, a finding which contrasted with the lack of correlation between DSH and IFN- γ production in the long-term graft survivors in the retrospective study. There was no correlation between graft outcome or DSH and IL-10 production in both arms of the study. At one year post-transplant, it may be possible to identify cadaveric recipients who have a lower risk of adverse events by the detection of DSH and low donor-specific IL-2 and high IL-4 production. In living-donor allograft recipients, good graft function and low donor-specific IL-2 production may be useful prognostic indicators.

CHAPTER 7: GENERAL DISCUSSION

INTRODUCTION

The incidence of end-stage renal failure is increasing and hence the demand for renal transplantation is also increasing each year. As more powerful immunosuppressive agents have been introduced and graft survival has improved, the incidence of complications is likely to increase further due to longer cumulative exposure to immunosuppression. Nevertheless, in some non-compliant patients and in some of those with malignancy, excellent graft function can be maintained despite little or no immunosuppression. This suggests that it may be possible to reduce the amount of immunosuppression in some recipients without inducing rejection episodes and it would be useful to be able to identify such patients.

Donor-specific hyporesponsiveness is a tolerance-like state that develops in some allograft recipients and has been demonstrated in patients with and without maintenance immunosuppressive therapy. The main objective of my study was to determine whether immunological methods could assist in identifying suitable renal transplant recipients for the reduction of immunosuppression. To achieve this objective, I examined whether good graft outcome was associated with the presence of DSH and a particular pattern of donor-specific cytokine production in long-term graft survivors. I then went on to determine whether similar immunological features could be found in patients who developed DSH within the first year post-transplant to assess the optimal time for reducing immunosuppression.

STUDY DESIGN AND METHODS

As long-term follow-up was not feasible during my research period, I chose to perform a retrospective study of long-term graft recipients to determine if there was any correlation between DSH or donor-specific cytokine production and graft outcome. This arm of the study comprised sixty patients, forty-six cadaveric (CAD) and fourteen living-related (LRD) recipients. These patients were selected on the basis of graft function and rejection history to represent the two ends of the spectrum of clinical outcome. The cadaveric group also included patients who had maintained their grafts on low-dose immunosuppression after developing a solid organ malignancy post-transplantation. The prospective arm studied the clinical outcome and immunological features of eighteen patients, fourteen CAD and four LRD recipients, at four time-points over the first year post-transplantation. As approximately 80% of transplants performed in the West of Scotland each year are derived from cadaveric donors, it is not surprising that cadaveric recipients represented 77% of both of my study populations.

I then compared the findings from the two arms of the study to determine whether patients with good graft outcome showed similar immunological features which might be used as a guide in selecting patients for reduction of immunosuppression. As described in Chapter 1, many in vitro techniques have been used to assess the donor-specific immune responses of transplant recipients. In my study, the MLR was used to detect DSH, as it has been shown to correlate with favourable graft outcome (Bas et al, 1993; Reinsmoen et al, 1993; Ghobrial et al, 1994; Kerman et al, 1997; Creemers et al, 1997) and it is the only technique which has been used in the selection of patients for reduction of immunosuppression

(Fletcher et al, 1984, Kahan et al, 1989, Kerman et al, 1997). This method is also easier and quicker than limited dilution assays, particularly when analysing large numbers of patients. As there is no consensus on the method used to define DSH as measured by MLR, I decided to use the relative response index, as it is a simple method that provides a single value which takes both donor reactivity and responses to third party control into account.

When I began my work, I used an RRI of $\leq 20\%$ to define DSH, a value used both by other investigators (Colombe et al, 1989; Ghobrial, 1994; Kim, 1996; Salomao et al, 1998; Ishido, 1999) and by the local Tissue Typing Laboratory in the selection of bone marrow donors. However, by the end of the study of the cadaveric cohort, I became aware that this may not have been the best value to show the discriminatory power of the MLR as a diagnostic test for detecting DSH. Therefore, I constructed a receiver operating characteristic curve, using the method described by Altman et al (1994), to determine which RRI was the best cut-off for defining poor graft outcome. When all values were considered, the highest sensitivity of the assay in detecting patients with a good graft outcome was 89% when the cut-off value of RRI was 28% or above. The highest specificity of the assay in detecting patients with a poor graft outcome was 94% when the cut-off value of RRI was 10% or below. As the cut-off level of RRI increased, sensitivity of the assay increased and specificity decreased, but the specificity was identical when a cut-off of 20% and 28% was applied. However, the sensitivity of the assay increased from 67% when a RRI of 20% was used, to 89% at a cut-off of 28%.

The predictive value of the assay was also assessed at each cut-off point. The positive predictive value of the assay in detecting the proportion of patients with a

good outcome was 67% using a cut-off of 20% compared with 73% when using a cut-off of 28%. The negative predictive value of the assay in detecting the proportion of patients with a poor graft outcome was 67% using a cut-off of 20% compared with 86% when using a cut-off of 28%. These results suggest that the sensitivity, specificity and predictive power of the MLR in detecting DSH were greater when a cut-off of 28% was used. The cadaveric cohort was therefore re-analysed using an RRI of 28% as the definition of DSH and this was subsequently applied to all patients studied.

LIMITATIONS OF STUDY

The data presented in my study should take into account several limiting, albeit frequently unavoidable factors in the study design and methodology.

STUDY DESIGN

The cadaveric cohort in the retrospective arm of the study was designed to compare patients with clinical features of good compared with poor graft outcome. The criteria used to define the outcome groups were subjective and an arbitrary level of serum creatinine ($< 140 \mu\text{mol/L}$) was used to define good graft function. The study size was limited further by the fact that sufficient donor material was not available for all the patients who met these criteria in the transplant database. Patients with poor graft function were difficult to recruit, and the number of patients was further restricted by the fact that many had lost their grafts due to chronic rejection. The patients with poor graft outcome were also relatively heterogeneous, as this group

included patients with late, multiple or steroid resistant acute rejection episodes, although chronic rejection was the main cause of graft dysfunction. Uraemia has been shown to impair lymphocyte function (Frymoyer et al, 1985; Kurz et al, 1986) and may have affected the proliferative responses in patients with poor graft function. However, I found that patients with impaired graft function had proliferative responses to third party and PHA similar to those in patients with good graft function, although both these groups had generally lower responses than non-immunosuppressed controls.

As only 10-20 living-related transplants are performed each year in the West of Scotland, the number of patients in this group was relatively small compared with the cadaveric pool in both arms of the study. The number of long-term living-donor recipients was further limited by the availability of the donor, some of whom were difficult to trace, or had died. Only six living-related transplants were performed during the recruitment period of my prospective study, and as two of these grafts failed, the study group was small and sub-group analysis could not be performed.

Immunological studies involving transplant patients are complicated by the use of immunosuppression. As it is unethical to reduce or stop treatment for the purpose of laboratory tests, I attempted to minimise the effects of immunosuppressants by taking all blood samples before the morning dose of cyclosporin or tacrolimus. The pre-transplant sample in the prospective cohort was also obtained before the first dose of immunosuppression. Any changes in immunosuppressive therapy were recorded but it is possible that these changes may have influenced immune responses.

METHODS

I performed a number of experiments to determine the best choice for a third party control. Previous studies have used a variety of such controls, including peripheral blood lymphocytes from random unrelated HLA-DR mismatched volunteers, or unrelated volunteers with a degree of HLA-mismatch similar to that in the recipient-donor pair, or a panel of individual or pooled volunteers. Alternatively, a panel or pool of donor splenocytes has been used as stimulators. As my work was not carried out in a tissue-typing laboratory, I could not use a source of third party controls that required known HLA-disparities, or which required large numbers of donor cells. Therefore, I used a pool of unrelated volunteers to represent a 100% reference control to which all recipients were tested. This allowed for all patients to be tested against the same easily obtainable third party controls, but the main disadvantage was that an MLR against pooled stimulator cells does not reflect exactly the MLR response against the donor, as more HLA-DR antigens are present to stimulate recipient cells.

Although I was able to test the reproducibility of the MLR in some patients, I could not verify my results for all patients as donor material was limited. However, I assessed the viability of donor cells before setting up the MLR in all patients to reduce the possibility of erroneous results. Additionally, I assessed the proliferative response of a volunteer against donor cells as a test of their functional viability.

RETROSPECTIVE STUDY

CADAVERIC AND LIVING-RELATED COHORTS

The cadaveric group without malignancy which consisted of thirty-six patients, provided the main foundation of the retrospective study. Despite many similar clinical features, such as age and duration since transplantation, patients who developed DSH were better matched at the HLA-DR locus than patients who did not develop DSH. This is consistent with the fact that alloreactive Class II MHC restricted CD4⁺ T cells play the most important role in mixed lymphocyte cultures (Reinsmoen, 2002). Although many previous studies which explored DSH following cadaveric renal transplantation did not state the degree of HLA-match, and Reinsmoen et al (1993) reported no correlation between DSH and the degree of HLA-DR match, Ghobrial et al (1994) reported a higher frequency of HLA-DR compatibility in association with DSH, although this was not statistically significant.

Overall, 61% of the cadaveric recipients in my study had DSH when examined at a median time of 9.8 years post-transplant. The longest prospective follow-up study reported previously was only of 4 years duration and found an incidence of DSH of 32% only (Kerman et al, 1997). As it has been shown that there is a trend towards lower donor-specific MLR responses in patients transplanted for 8-12 years compared with those transplanted for 2-5 years (Thomas et al, 1977), this may explain the higher incidence of DSH in my cohort compared with that of Kerman et al.

In comparison, 57% of the living-related recipients in my study had DSH at median time of 5.7 years post-transplant. All of the HLA-ID patients and 38% of the HLA-HI showed DSH. DSH was not found in the HLA-NI recipient, despite a good

graft outcome. My findings are consistent with reports of DSH in 95-100% of HLA-ID recipients and 31-47% of HLA-HI recipients within the first two years post-transplant (Fletcher et al, 1984; Kahan et al, 1989; Kerman et al 1997). However, one other study has reported DSH in 75% of HLA-HI recipients (Kim et al, 1996). This discrepancy may be explained by the selection criteria used by Kim et al, who included only patients with good graft function who had no history of acute rejection and had been transplanted for up to 8 years. I could find no reports on the incidence of DSH in HLA-NI recipients.

Although I found a higher incidence of DSH in both cadaveric and living-related recipients with a good graft outcome compared with those with a poor graft outcome, there was a significant correlation between DSH and graft outcome only in the cadaveric cohort. Other groups have shown an association between DSH and better long-term graft survival in both cadaveric (Reinsmoen et al, 1993; Ghobrial et al, 1994; Kerman et al, 1997) and living-related (Reinsmoen et al, 1993; Kim et al, 1996; Kerman et al, 1997; Salomao et al, 1998) recipients. The lack of a significant correlation between DSH and graft outcome in my living-related cohort may reflect good HLA-matching even among the patients with poor graft outcome, as well as the greater heterogeneity of my small group compared with other series.

Not surprisingly, I found that both cadaveric and living-related recipients with a good graft outcome had significantly lower levels of serum creatinine than patients with a poor outcome, even though serum creatinine was not one of the criteria used to define poor graft outcome. In the cadaveric cohort, I found a significantly lower serum creatinine in patients with DSH compared with those without DSH and the RRI correlated significantly with serum creatinine levels.

These features were not observed in the living-related group who consisted of a smaller group with shorter duration of graft function. In contrast, previous reports have shown no correlation between DSH and the level of graft function as measured by serum creatinine in cadaveric recipients (Reinsmoen et al, 1993; Ghobrial et al, 1994), although one of these studies (Reinsmoen et al, 1993) did report lower mean serum creatinine levels in living-related recipients. Again, the discrepancy between my study and these earlier ones may be the differences in time after transplant, as well as the small size of my living-related cohort.

The overall incidence of acute rejection in my cadaveric recipients who showed DSH was similar to those who did not (50% versus 43%), but the incidence of rejection was significantly lower in living-related recipients who showed DSH compared with those who did not (44% versus 100%). As discussed in Chapter 1, the timing of acute rejection episodes is an important factor and acute rejection after the first three months post-transplant has been shown to have the greatest impact on graft survival (Joseph et al, 2001; Sijpkens et al, 2003). Thus, when early steroid-responsive rejection episodes were excluded from my analysis, DSH was associated with fewer late and multiple acute rejection episodes in both cadaveric and living-related recipients. This is consistent with the findings of other groups which showed a reduced incidence of late acute rejection in patients with DSH (Reinsmoen et al, 1993; Ghobrial et al, 1994; Kerman et al, 1997; Creemers et al, 1997).

DSH was not confined to patients with good graft function, as it was also found in two patients with late or recurrent acute rejection episodes, as well as in four cadaveric and two living-related recipients with chronic rejection. This supports other reports which have shown that chronic rejection may be found in some patients

who show DSH (Reinsmoen et al, 1994; Bohmig et al, 2000) and suggests that chronic rejection may not be entirely immune mediated. More recently, it has been shown that the risk of chronic rejection in patients who show DSH is higher among those patients who have experienced acute rejection compared with those who have been free of acute rejection (Reinsmoen et al, 2002). However, patients who experience acute rejection have a higher risk of chronic rejection if they remain responsive to their donor compared with those who develop DSH. Together these results suggest that DSH is not exclusive to patients with a good graft outcome and that the risk of chronic rejection is lower in patients who develop DSH.

My studies of donor-specific cytokine production also gave discordant results in the two groups of recipients. In the cadaveric cohort, DSH and good graft outcome were associated with the production of low levels of donor-specific IL-2 and high levels of IL-4 and there was a significant correlation between the RRI and donor-specific IL-2, IL-4 and IFN- γ production. In the living-related cohort, DSH did not correlate with donor-specific cytokine production, good graft outcome was associated only with low levels of donor-specific IL-2, and there was no correlation between RRI and cytokine production. In fact the living-related group, particularly HLA-ID recipients, produced low levels of all cytokines in response to donor stimulation, which again may reflect the high degree of HLA-compatibility in this group. The level of immunosuppression in patients who developed DSH and those who did not was similar in both cohorts and DSH itself was not due to generalised immune suppression, as all patients showed good proliferative responses and cytokine production in response to stimulation with third party cells and PHA.

The correlation between the donor-specific proliferative response and IL-2 production in cadaveric recipients was not unexpected, given the role of IL-2 as a T cell growth factor. However, IL-2 also appears to be essential for tolerance by facilitating the deletion of alloreactive T cells through activation-induced cell death (Zheng et al, 2000) and the development of regulatory T cells in the periphery (Furtado et al, 2002; Li et al, 2003). For these reasons, there is now concern that immunosuppressive agents that inhibit IL-2 production, such as cyclosporin and tacrolimus, may block tolerance. This is supported by the findings that the administration of high dose calcineurin inhibitors at the time of tolerance induction prevents long-term graft survival (Larsen et al, 1996; Kirk et al, 1999) and the development of CD4⁺ CD25⁺ regulatory cells (Wood et al, 2003). Although rapamycin interferes with IL-2 signalling, it does not appear to have a similar effect on AICD, although its effect on regulatory cells remains unclear (Wood et al, 2003). My data are consistent with the idea that the level of donor-specific IL-2 may have complex effects on the allospecific immune response, with high levels stimulating clonal expansion and low levels facilitating tolerance.

The negative correlation between the relative response index and IL-4 production suggests that IL-4, and/or IL-4 dependent cells, may play a role in the maintenance of donor-specific tolerance. These findings are consistent with other evidence for IL-4 producing regulatory T cells in allograft tolerance induced by donor-specific transfusion (Yang et al, 1998) and with the fact that tolerance-inducing regimens often suppress IL-2 but not IL-4 gene transcription (Nickerson et al, 1993; Chen et al, 1996; Mottram et al, 1995; Onodera et al, 1997). Furthermore, infectious tolerance in which CD4⁺ T cells can adoptively transfer specific tolerance

to a naïve host by suppressing the ability of host cells to effect allograft rejection, appears to be driven by IL-4-dependent immune deviation (Davies et al, 1996; Bushell et al, 1999; Ke et al, 2000). IL-4 may facilitate allograft tolerance either by promoting Th2 differentiation with a resultant decrease in inflammatory Th1 activity, or by acting as a growth factor for regulatory T cells (Li et al, 1999; 2003).

The significant correlation between the degree of donor responsiveness and IFN- γ production in cadaveric recipients suggests a possible correlation between a poor allograft outcome and a Th1 profile. IFN- γ is generally regarded as a pro-inflammatory cytokine and increased intra-graft IFN- γ expression is frequently detected in rejecting allografts, while decreased expression is found in long-term surviving grafts (Takeuchi et al, 1992; O'Connell et al, 1993; Sayegh et al, 1995). Although some reports suggest that IFN- γ may have a protective role after transplantation, as the administration of anti-IFN- γ antibody has been shown to lead to rapid allograft rejection (Markees et al, 1998; Konieczny et al, 1998), a recent study supports my findings by showing that HLA-DR allopeptide-specific T cell clones generated from recipients with chronic rejection secrete IFN- γ , while clones from recipients with stable graft function produce IL-4 and IL-10 (Waaga et al, 2001).

An important finding of my study was that it failed to support the hypothesis that the production of inhibitory cytokines such as IL-10 and TGF- β is associated with DSH. I found that both cadaveric and living-related recipients with good graft outcome appeared to produce lower levels of donor-specific IL-10 and there was no significant correlation between IL-10 production and donor responsiveness or graft outcome in either cohort. The immunosuppressive properties of IL-10 are well

documented, as it can inhibit antigen presentation and CD4⁺ T cell proliferation in vitro, as well as the generation of allo-specific cytotoxicity by virtue of its effects on the costimulatory function of APC (Groux et al, 1998). However, IL-10 has also been shown to have pro-inflammatory properties depending on the nature of the APC and the local microenvironment. IL-10 does not block the initiation of an alloimmune response by allogeneic DC and B cells (Nickerson, 1997). IL-10 also augments IL-2 supported proliferation of CD8⁺ T cells, thereby enhancing cytotoxicity (Chen et al, 1991; Groux et al, 1998). Therefore, IL-10 may be beneficial or deleterious following transplantation. IL10 appears to have a dose-dependent effect on acute GvHD lethality after bone marrow transplantation with both reduction (Baker et al, 1999; Holler et al, 2000) and exacerbation (Hempel et al, 1997; Blazar et al, 1998) of GvHD being reported. IL-10 or IL-10 mRNA expression has also been associated with acute rejection in some transplant models (Zheng et al, 1995; Xu, et al, 1995; Qian et al, 1996; Lang et al, 1996). Together, my results suggest that the regulatory mechanism underlying DSH may be independent of IL-10 and this could be tested by assessing donor-specific proliferation in the presence of anti-IL-10 or anti-IL-10R antibody in patients who showed DSH.

The role of TGF- β in allograft acceptance and chronic rejection has been of great interest in recent years. Although currently viewed as an immunoregulatory cytokine (Letterio et al, 1998; Josien et al, 1998; Bickerstaff et al, 2000), TGF- β also has pro-inflammatory and fibrogenic properties (Border et al, 1994; Hutchinson, 1999; Blobe, 2000). I found that good graft outcome in living-related recipients was associated with lower levels of donor-specific TGF- β production compared with patients with poor graft outcome and there was no correlation between DSH and

donor-specific TGF- β production. In contrast, two groups have demonstrated TGF- β dependent suppression of donor-specific DTH responses in renal transplant patients who maintained good graft function without immunosuppressive therapy (Van Buskirk et al, 2000; Lee et al, 2002). I also found that patients with chronic rejection produced lower levels of donor-specific TGF- β than patients without chronic rejection. In contrast, other studies have demonstrated that intra-graft TGF- β mRNA correlated with interstitial fibrosis and chronic allograft nephropathy (Sharma et al, 1996; Suthanthiran et al, 1998). Additionally, Brenchley et al (1998) proposed local activation of TGF- β may be a more important determinant of subsequent graft fibrosis than the actual level of TGF- β as prolonged over-expression of TGF- β in the vessels or interstitium of the graft may contribute to chronic rejection. Therefore it is possible that the systemic production of TGF- β I measured may not reflect activities within the graft even in patients with established chronic rejection.

Although I found no evidence to support a role for TGF- β in regulating donor-specific immunity, I did find an association between TGF- β production and immunosuppressive therapy. Patients treated with tacrolimus produced higher levels of donor-specific TGF- β than cyclosporin treated patients, while patients on neither drug produced almost undetectable levels of TGF- β . The effects of these drugs on TGF- β production are controversial, as Shin et al (1998) reported that cyclosporin enhances TGF- β production, while others found no evidence that cyclosporin (Hughes et al, 1999; van der Mast, 2000) or tacrolimus (Hughes et al, 1999) induce TGF- β production. As the measurement of TGF- β is technically difficult, these conflicting results may be explained by differences in methodology with variable

results reported using different immunoassays (Kropf et al, 1997). I measured active TGF- β using a commercial ELISA kit, but analysed only a limited number of patients and it would have been interesting to assess TGF- β production in the cadaveric cohort where larger numbers of patients were available.

A criticism of many studies of cytokine production in transplantation has been the use of PCR to assess cytokine gene expression in graft infiltrating cells, a method which may not reflect cytokine protein production. Therefore, I performed PCR studies in some patients to determine whether cytokine gene expression correlated with donor-specific cytokine protein secretion. I used a non-quantitative RT-PCR method, as I wished to simply establish whether the cytokine gene was being expressed or not. However, this proved to be inconclusive, particularly when TGF- β was assessed, as gene expression was often detected in autologously stimulated cells as well as those in MLR. Furthermore, I could not achieve a reliable PCR method for detecting IL-4 mRNA, despite trying a number of different primers. Most importantly, the PCR and ELISA data were frequently discordant and it might be useful to use a quantitative PCR method so that donor-specific cytokine gene expression could be related directly to control levels and to ELISA data.

To date, only a few case studies have reported the cytokines produced by patients who show MLR-defined DSH, all of whom were living-related renal transplant recipients maintained on no immunosuppression due to malignancy or because of non-compliance. Kusaka et al (1995) demonstrated high donor-specific IL-4 production in a patient who received pre-transplant donor-specific blood transfusions, while Ishido et al (1999) demonstrated lower levels of IFN- γ and higher levels of IL-4 after donor stimulation than after third party stimulation. Christensen

et al (1998) showed reduced mRNA expression of IL-2 and IFN- γ in a patient with post-transplant lymphoproliferative disorder. A more detailed report of intra-graft cytokine production in cadaveric renal transplant recipients showed low levels of both IL-2 and IL-10 in patients with stable graft function, high levels of IL-2 in patients with acute rejection, and high levels of IL-10 and low levels of IL-2 in patients with chronic rejection (Oliveira et al, 1998). In studies of liver transplant patients, increased IL-2 and IFN- γ production have also been found to be associated with acute rejection (Minguela et al, 1999), while IL-4 and IL-10 were associated with stable graft function (Chung et al, 1998). Together, these reports support my findings of high donor-specific IL-4 and low IL-2 production in association with DSH and good graft outcome, as well as my finding of high donor-specific IL-10 production in patients with chronic rejection.

MALIGNANCY COHORT

Ten cadaveric recipients who developed a de-novo solid organ malignancy after transplantation were studied to assess the effect of immunosuppressive therapy and to determine whether DSH occurred in patients who maintained their grafts on low-dose immunosuppression. Four of these patients maintained excellent graft function only with prednisolone mono-therapy, while the other patients received low-dose dual therapy. Significantly fewer patients in the malignancy group received cyclosporin or azathioprine compared with cadaveric recipients without malignancy, and none of the patients with malignancy were being treated with tacrolimus or mycophenolate at the time of the study. Nevertheless, the patients with malignancy shared many clinical features with the good outcome group of the cadaveric cohort

without malignancy, including good graft function, a low acute rejection rate and a similar incidence of DSH.

The malignancy cohort also shared some immunological features of patients with good graft outcome without malignancy, with both groups producing significantly lower levels of IL-2 in response to donor stimulation compared with patients with a poor graft outcome. However, low levels of donor-specific IL-4 were found in patients with malignancy in contrast to the high donor-specific IL-4 production I found in cadaveric recipients without malignancy with good graft outcome. A striking finding in the malignancy group was the production of approximately four-fold higher levels of IL-10 in response to donor stimulation than any other cytokine. IL-10 has been implicated in both the development of EBV-associated post-transplant lymphoproliferative disorder (PTLD) and the induction of a state of operational tolerance in patients who maintained their grafts after discontinuation of immunosuppression (Birkeland et al, 2000). MLR-defined DSH was found in 4/5 of these patients, although chronic rejection developed in two of these patients after successful treatment for PTLD. As EBV encodes a polypeptide analogous in structure and bioactivity to human natural IL-10, viral IL-10, and the ELISA assay detects both sources of IL-10, it is difficult to determine the source of IL-10 in these patients. Furthermore, as IL-10 levels increased prior to the diagnosis of PTLD and fell after cancer treatment, it is possible that viral IL-10 may account for these findings. However, in view of my previous results in patients without malignancy, it is unlikely that high donor-specific IL-10 production itself could explain the development of DSH in my sub-group of patients with malignancy. In addition, as I found low levels of IL-4 in my malignancy cohort, it is possible that

other factors might be important in maintaining DSH and it would have been interesting to evaluate TGF- β production in these patients for evidence of immunoregulation.

Although there are a number of possible mechanisms for the development of malignancy post-transplantation, CD4⁺CD25⁺ T cells which are essential in maintaining self-tolerance, may play an important role in the down-regulation of tumour immunity (Tanaka et al, 2002; Wood et al, 2003). These cells may act to impede the generation of effective immunity against autologous tumours and host responses have been shown to be augmented by the depletion of these cells (Shimizu et al, 1999; Tanaka et al, 2002). CD4⁺CD25⁺ T cells appear to mediate their suppressive effects by a cytokine-independent and cell contact dependent mechanism, therefore their activity is likely to be independent of IL-10. These regulatory cells might also help to explain the high frequency of DSH that I found in my malignancy cohort, which could have been mediated by the same mechanism.

PROSPECTIVE STUDY

Most previous reports on DSH have been prospective studies which compared pre-transplant donor-specific responses with those 6-51 months post-transplantation, although most studies ended at 12 months after transplantation (Bas et al, 1992; Reinsmoen et al, 1993; Ghobrial et al, 1994; Creemers et al, 1997; Kerman et al, 1997). Frequent serial laboratory analyses over a prolonged period is the ideal way to explore this group, but this is rarely feasible in practice due to financial and time restraints, and I was only able to perform the immunological assays for up to one

year after transplant. However, the clinical course of patients was followed for 2 years post-transplantation to determine whether stable graft function was maintained and I also considered that my period of 1-year follow-up was appropriate as any prognostic laboratory test for tailoring immunosuppression would be of greatest value within this time.

My study population consisted of eighteen renal allograft recipients (14 cadaveric and 4 living-donor), all of whom received standard triple immunosuppressive therapy. At the end of the first year post-transplant, 11/18 patients (9 cadaveric and 2 living-donor) met the clinical criteria for good graft outcome that I had defined in the retrospective study. Four patients (3 cadaveric and 1 living-donor) met the clinical criteria for poor graft outcome, and the remaining three patients did not meet the pre-determined criteria for either outcome group. The acute rejection rate appeared to be higher among living-donor than cadaveric recipients (75% versus 33%), but this is probably due to the small size and heterogeneity of the living-donor group, which consisted of only one HLA-ID, two HLA-HI and one NI recipients.

I found that three cadaveric recipients, who all received well-matched grafts and had no acute rejection episodes, showed a $RRI \leq 28\%$ immediately pre-transplant, but only one of these patients continued to show DSH at one year post-transplant. As the level of RRI in the two patients who did not show DSH was 30% and 32% at 12 months post-transplant, it is possible that random fluctuations in donor-responsiveness are responsible for this finding, and it would have been interesting to follow them for a longer period to determine whether they would develop DSH with a $RRI \leq 28\%$ in the future. Such fluctuation were seen in most of

my patients and as all samples for each patient were analysed simultaneously, they may, at least in part, be attributable to the changes in immunosuppressive therapy which occurred during the course of my study. Similar fluctuations were also observed by another group who analysed patients serially (Ghobrial et al, 1994).

I found DSH in three cadaveric recipients at three months after transplant, in four patients at six months and in five patients at 12 months, and DSH was maintained in all these patients throughout the study period. In previous prospective studies of cadaveric recipients, DSH has been detected as early as three months post-transplant (Reinsmoen et al, 1990; Bas et al, 1992), although another group found that the mean time to the development of DSH was 21 ± 9 months post-transplant (Kerman et al, 1997). The only living-donor recipient who developed DSH in my study received an HLA-ID graft and showed an $\text{RRI} \leq 28\%$ immediately pre-transplant which was maintained throughout the first year post-transplant. This is in keeping with previous reports that 95-100% of HLA-ID recipients showed DSH prior to transplantation (Kahan et al, 1989; Kerman et al, 1997).

At the end of the first year post-transplant, DSH was found in 6/18 (33%) patients overall, comprising 5/14 (36%) cadaveric recipients and 1/4 (25%) living-donor recipients. As outlined in Chapter 1, the reported incidence of DSH in cadaveric recipients is variable, but groups who have assessed MLR-defined DSH over a period of 1 year, as I did, have found the incidence to range from 25% (Reinsmoen et al, 1993) to 58% (Ghobrial et al, 1994). This wide variation may be attributable to the use of homozygous typing cells (Reinsmoen et al, 1993) rather than the original donor cells (Ghobrial et al, 1994) as stimulators in MLR. An incidence of 56% using donor cells at 2 years post-transplant has also been reported

(Creemers et al, 1997). Although the incidence of DSH in HLA-HI recipients has been reported to be 27%-47% at 12 months post-transplant (Reinsmoen et al, 1993; Kerman et al, 1997), neither of the HLA-HI recipients in my study group showed DSH.

In contrast to my findings in the retrospective cadaveric cohort, I found no significant difference in serum creatinine levels in the prospective study between patients who showed DSH and those who did not throughout the first two years post-transplant. However, as many others have reported (Grino et al, 1990; Reinsmoen et al, 1993; Ghobrial et al, 1994; Kerman et al, 1997; Creemers et al, 1997), the patients in my prospective cohort who developed DSH experienced significantly fewer acute rejection episodes than patients who did not (17% versus 58%). However, one patient who showed DSH developed biopsy-proven chronic rejection, as well as one patient who did not show DSH. At the end of the first year post-transplant, only 3/6 patients (all cadaveric recipients) who showed DSH had good graft function and all continued to maintain excellent graft function at two years post-transplant.

Donor-specific cytokine production has not been assessed previously during the development of DSH, which would be important to show if DSH is indeed associated with a shift from Th1 to Th2 cytokine production as is widely speculated. It would also be important to assess how early the production of cytokines may predict graft outcome. At all time points I examined, patients who developed DSH produced lower levels of donor-specific IL-2 than patients who did not develop DSH, although this only reached statistical significance immediately pre-transplant. Both patients with and without DSH produced higher levels of IL-2 in response to third party than donor stimulation at all time points. The DSH group also produced lower

levels of donor-specific IFN- γ throughout the 12-month study period compared with those who did not develop DSH, but these differences were only statistically significant at 6 and 12 months post-transplant. IFN- γ production was higher after third party than donor stimulation in the DSH group but higher after donor than third party stimulation in the non-DSH group at all time points. These findings are generally consistent with the significant correlation between RRI and donor-specific IL-2 and IFN- γ production I found in the retrospective group.

In the prospective cohort, donor-specific IL-4 production generally rose throughout the study period, but the DSH group appeared to produce higher levels of IL-4 pre-transplant and at 12 months after transplant than the non-DSH group. This is in keeping with the association between DSH and high donor-specific IL-4 production and the correlation between RRI and donor-specific IL-4 production that I found in long-term cadaveric graft survivors of the retrospective cohort. In addition, the patients with good graft outcome produced lower levels of IL-2 and higher levels of IL-4 in response to donor stimulation, as I found in the cadaveric cohort. As noted earlier, IL-10 producing regulatory T cells have been implicated in transplant tolerance (Hara et al, 2001; Kingsley et al, 2002), but I found no correlation between DSH and IL-10 production in either arms of my study.

The consistent finding in both arms of my study was low donor-specific IL-2 and high IL-4 production in cadaveric recipients with DSH who maintain good graft function after one year post-transplant and in long-term graft survivors. This may reflect good HLA-matching, as HLA-DR compatibility was a significant factor in the retrospective cadaveric group and five of the six patients in my prospective study who developed DSH had no mismatches at the HLA-DR locus. The low production

of IL-2 in patients who showed DSH could not be attributed to treatment with calcineurin inhibitors, as similar numbers of patients who did not show DSH were treated with cyclosporin. As discussed above, IL-4 may be required to maintain DSH by promoting the development of Th2-like regulatory cells, while low levels of donor-specific IL-2 may facilitate apoptosis of alloreactive cells and the development of regulatory T cells, thereby providing a balance between regulation and deletion of alloreactive responder cells.

TAILORING OF IMMUNOSUPPRESSION

The benefits of reduction of immunosuppression must be weighed against the risk of acute rejection and potential graft loss. The optimal time for reduction of immunosuppression is unknown, but as I found fluctuations in the RRI within the first 12 months post-transplant and the risk of acute rejection is highest during this early period, tailoring of immunosuppression may be best considered at one year post-transplantation. Although clinical factors play an important role in the decision to tailor immunosuppression, they are of limited value as precise predictive factors (Schulak et al, 1990; Hricik et al, 1993; Silkensen et al, 2001). Good graft function does not always imply low donor responsiveness and an isolated raised serum creatinine level is not necessarily an accurate guide, as it is sometimes a late indicator of graft dysfunction and can be affected by non-immunological factors. The ultimate aim of my work was to determine whether the assessment of donor-specific cytokine production in stable renal transplant recipients who show DSH would be of value for tailoring immunosuppression.

Many groups have suggested that the testing of T cell reactivity could be used to select patients for reduction of immunosuppression, but did not test this hypothesis directly (Reinsmoen et al, 1993; Zanker et al, 1993; Ghobrial et al, 1994; DeBruyne et al, 1995; Creemers et al, 1997). Furthermore, the few studies that have assessed the effects of reducing immunosuppression on donor-specific cell reactivity have produced conflicting results, as some reports have shown a good correlation between clinical outcome and in vitro responsiveness (Goulmy et al, 1991; Beik et al, 1997; van Besouw et al, 2000), but others have failed to do so (Mazariegos, et al, 1995; Creemers et al, 1998). The lack of consistency in these studies may be due to differences in the methods used to assess donor reactivity and in the duration of patient follow-up. To date, only one centre has used MLR-defined DSH to select patients for reduction of immunosuppression, but this group reported acute rejection episodes and graft losses even among HLA-ID recipients (Fletcher, 1984; Kahan, 1989; Kerman, 1997).

I have extended these earlier studies by performing retrospective and prospective studies to determine whether the analysis of donor-specific cytokine production would be of additional value to the detection of DSH by MLR and to determine whether this could be applied to both cadaveric and living-related recipients. In the retrospective study, I found that cadaveric recipients who showed DSH produced approximately two-fold higher levels of donor-specific IL-4 than IL-2 irrespective of the level of graft function and that patients who did not show DSH produced a low IL-4/ IL-2 ratio, even in the presence of good graft function. These findings suggested that the production of a high donor-specific IL-4/ IL-2 ratio was more in keeping with the presence of DSH than the level of graft function, and that

graft function may be influenced by other factors. However, of the sixteen long-term cadaveric recipients I identified with DSH and good graft function, only nine patients produced higher levels of IL-4 than IL-2 after donor stimulation and the ratio of donor-specific IL-4/ IL-2 production in these patients was 3.7. In the prospective arm of the study, I found four-fold higher levels of donor-specific IL-4 than IL-2 only in the three cadaveric recipients with DSH who maintained good graft function at 12 months post-transplant. Therefore, the consistent finding in both arms of my study is the detection of a high donor-specific IL-4/ IL-2 ratio in patients with DSH and good graft function, but this applied to only cadaveric recipients. On the basis of my results, it could be suggested cadaveric recipients who showed DSH and maintain good graft function with no late acute rejection episodes, could be considered for reduction of immunosuppression if they produce approximately four-fold higher levels of IL-4 than IL-2 after 12 months post-transplant.

The utility of cytokine analysis in living-related recipients may be limited. Low donor-specific IL-2 production was associated with good graft outcome in the retrospective cohort, but this could not be confirmed in the prospective cohort. Furthermore, I found DSH and good graft outcome in six patients, five of whom were recipients of HLA-ID grafts, but I found no association between DSH and cytokine production in either arm of my study. Therefore, the degree of HLA-compatibility appears to an important factor in the development of DSH and graft outcome. HLA-NI recipients are unlikely to be good candidates for reduction of immunosuppression, as the risk of acute rejection would outweigh the potential benefit, unless under exceptional circumstances such as malignancy.

I conclude that there is unlikely to be a single immunological test or clinical parameter that will allow safe reduction of immunosuppression. As previous studies have suggested, the detection of DSH as judged by poor proliferation in MLR, is not alone sufficient to predict long-term graft outcome and to guide clinical decisions, even among HLA-ID living-related recipients. However, my results do suggest that the presence of DSH, together with low donor-specific IL-2 and high IL-4 production in the presence of stable graft function at one year post-transplant, may be indicators of cadaveric recipients who are at low risk of adverse events. In living-related recipients, patients who receive HLA-ID grafts are the best potential candidates for tailoring of immunosuppression, as DSH is usually found and good outcome is associated with low donor-specific IL-2 production.

FUTURE WORK

My study has probably given rise to more questions than answers, but perhaps the most glaring unanswered questions are whether regulatory cells or inhibitory cytokines, such as IL-10 and TGF- β mediate DSH. Certainly, I was unable to provide evidence of a correlation between DSH and donor-specific TGF- β production, as analysis was limited to the living-donor retrospective cohort which generally produced low levels of all cytokines in response to donor stimulation. Therefore, I anticipate that it would be more useful to perform future work on cadaveric recipients.

I propose that a prospective study of first cadaveric graft recipients over 2 or more years with frequent serial immunological analyses, to detect DSH and measure

donor-specific IL-2, IL-4, IL-10 and TGF- β production by quantitative PCR and ELISA, would help to answer these questions. A quantitative PCR method would overcome some of the hazards of measuring TGF- β by ELISA, and provide useful confirmation of other ELISA results. It would be particularly interesting to see whether patients who produce low levels of donor-specific IL-2 and high levels of IL-4 pre-transplant would also have high IL-10 and TGF- β mRNA expression. Further work could focus on the nature of the cytokine-producing cells and sorting CD4⁺ cells for those that express CD25 as a marker of regulatory activity, in this model of tolerance.

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